Parasite epidemiological and pollution/parasite interaction studies on sticklebacks (Gasterosteus aculeatus L.) from the River Wandle.

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PARASITE EPIDEMIOLOGICAL AND POLLUTION/PARASITE INTERACTION STUDIES ON STICKLEBACKS (*GASTEROSTEUS ACULEATUS* L.) FROM THE RIVER WANDLE

BY

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A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Environmental Science of the University of London

1998

ABSTRACT

This thesis investigated a population of sticklebacks (***Gasterosteus aculeatus***) in the River Wandle, a tributary of the River Thames. The principal aspects of this multifaceted investigation related to patterns of parasitic infection in the wild stickleback population, possible associations between levels of parasitic infection and different degrees of pollution in sections of the river and experimental laboratory studies on the interactions between phenol, fish toxicity and fish parasitic loads.

A detailed descriptive epidemiological survey of stickleback hosts was made on parasitic infections in two contrasting regions of the river which exhibited characteristically different levels of infection with the plerocercoids of ***Schistocephalus solidus***. Seasonal changes in parasitisation were described and analyses were made of host condition factors in relation to parasitisation and associated levels of pollution in the two river regions.

Using *Schistocephalus* - infected and uninfected sticklebacks from the Wandle, laboratory experiments were carried out to characterise the possible relationships between cestode infection, toxicity and biotransformation of phenols in these hosts. These investigations suggested that parasitisation had no significant effect on the toxicity or phase II glucuronidation of phenols when compared with the situation in uninfected fish.

In a further attempt to identify any impact of parasite on toxicity and biotransformation of phenol in fish, parallel experiments were performed on control perch (***Perca fluviatilis***) and perch whose livers contained plerocercoids of the pseudophyllidean cestode ***Triaenophorus nodulosus***. Despite the extensive histopathology in the livers of such fish, no significant influences were discovered on the ***in vitro*** investigation.

The results of the field and laboratory studies have been discussed within the framework of host/parasite interrelationships and the suggested role of parasites as biological markers of pollution - induced stress in fish.

AKNOWLEDGMENT

I wish to express my grateful thanks to my two supervisors, Professor P. J. Whitfield and Dr. S. Smith for their advice and support during the period of my study.

I would also like to thank other members of the Division of life Science at King's college London for technical and other help, including Alan Maple, Reg Reed and Valerie Jordan.

My thanks are also due to Simon Partlam of the Environment Agency at Reading, who was extremely helpful in providing infected perch for the studies described in chapter 5.

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General Introduction

1. Ecosystem health, fish disease and aquatic environmental monitoring

1.1. Freshwater ecosystems, fish health and pollution

Freshwater environments constitute regions, including rivers, estuaries and lacustrine areas, of considerable ecological, recreational and commercial significance. They possess a number of features, relating to man's use, species present, climatic variables, salinity and physicochemical attributes which make them structurally and functionally diverse and thus provide habitats for a vast range of fish and invertebrate species. Anthropogenic influences, however often affect freshwater environments and these can lead to alterations in community and population structure, inputs of noxious chemical agents and the modulation of levels of disease(s) of bacterial, viral and parasitic origin.

Among the range of techniques for environmental monitoring in freshwater systems are some based on data derived from behavioural and physiological responses by fishes in response to a range of substituted aromatic compounds and pesticides. Using electrophysiological techniques, notably, electrical organ discharges (EOD), Campbell *et al* (1990) and Lewis *et al* (1990) have shown that EOD are proportionately linked to concentration of pollutants and that with incrementally increasing doses of phenol there is a concomitant increase in electrical output. Campbell *et al* (1990) and later Lewis *et al* (1990) indicated that electrical changes linked to alterations in olfactory gustatory and branchial movements could also be used constructively in establishing dose-response relations for compounds in the mormyrid fish (*Gnathonemus petersii*). Alazemi *et al* (1996) have further demonstrated that gross histopathological changes are also closely allied to levels of toxicant exposure. For example Alazemi *et al* (1996), have shown that heavy metals, cadmium (Cd) chromium (Cr) and copper (Cu)

cause degeneration of the secondary lamellae, sub-epithelial disruption and epithelial hyperplasia of fish gills. Certain indigenous species of fish, within the U.K., for example eel (*Anguilla anguilla* L.), flounder (*Platichthys flesus* L.), three-spined stickleback (*Gasterosteus aculeatus* L.), nine-spined stickleback (*Pygosteus pungitius* L.), and minnow (*Phoxinus phoxinus* L.) which are distributed in a widespread fashion throughout freshwater environments in the United Kingdom have potential value in analysing disease-pollution interactions (Overstreet, 1988, 1993; MacKenzie *et al* 1995).

Laboratory experiments and studies of wild - caught fish suggest that exposure to oil and its components reduces the prevalence and intensity of gut parasites (nematodes, Kiceniuk and Khan, 1983; protozoa and encysted helminths, Haensly *et al*, 1982), but may increase infection by gill parasites (monogeneans, Khan and Kinceniuk, 1988; trichodinid ciliates, Khan, 1990). Paperna (1975) reported that mullet *(Mugil cephalus*) may have died from increased monogenean infection (*Benedenia* sp.) subsequent to exposure from oil leaking from submarine oil wells. Also, Kiceniuk and Khan (1983) showed that leeches were directly affected by oil, the fish leech *Johanssonia arctica* was allowed to feed, then exposed for 69 days to a water - accommodated fraction of Venezuelan crude oil. Digestion was slower in oil - exposed leeches, and reproduction was poorer, with control leeches producing three times more eggs than exposed leeches.

It was suggested that fish occupying areas where pollutant stresses (both physical and chemical) had been recognised, were more susceptible to parasitism and infection due to immunosupressant effects, alterations in buccal and opercular cavity morphology and changes in internal organ function (Smith, 1969; Arme and Halton, 1972; Hawkes, 1977). These workers suggested that stressed fish harbour greater numbers of parasites, following exposure to xenobiotic agents, than fish from clean areas.

It is somewhat unfortunate that pollution-parasitism interactions were treated as rather obscure phenomena until the early 1980's when there was a greater appreciation of the effects of pollution on fish disease. Several reviews on environmental stress and disease in fishes specifically excluded parasitism in their consideration of

physiological stress (Pickering, 1981; Wedemeyer and Goodyear, 1984). Moller (1985), however, recognised the significance of parasitism in the context of physiological stress in fish and has indicated that it is one of the single most important factors in the incidence of diseases in feral and cultured fishes.

Since Moller's (1985) recognition of parasitism as a factor influencing physiological stress in fish there has been a rapid advancement in the state of knowledge in this field. Many attempts have been made since 1980 toward the greater incorporation of parasites as indicators of environmental pollution and disease in freshwater and marine fishes (MacIntyre and Pearce, 1980; Yevich and Barszcz, 1983; Moller, 1985, 1987a, and b; MacVicar, 1986). Initially, it was felt that parasities were not necessarily a reliable indicator of environmental quality since they lacked the sensitivity exhibited by biochemical and physiological indices.

MacVicar (1986) hinted that pathological changes in fish often occured over a prolonged time-course, and therefore, these changes may reflect a series of accumulative events and may not relate to short-term changes in the environment. More recently, MacKenzie *et al* (1995) have suggested that a more rapid response to pollution - induced physiological stress may be obtained from parasite models by closer investigation of transmission processes, more specifically, by examining the life-cycles of parasites in relation to environmental changes.

There are several valid reasons which suggest that many parasites have a useful role in assessing fish health and determining the impact of pollutants. Firstly, there are far more parasitic species than free-living organisms (May, 1988). Secondly, some parasites have complex life-cycles, involving several intermediate stages in the end leading to their transmission to the definitive host. Disruption of these complex and obligatory pathways by environmental changes such as pollution could have implications for the development of the parasite and therefore this could be used for purposes of monitoring environmental disturbance (Khan and Thulin, 1991). Considering the intermediate stages of a parasite life - cycle also widens the choice of potential environmental indicators.

Urban catchments often receive a variety of polluting inputs, including heavy metals, pesticides and aromatic hydrocarbons. The River Wandle, a sampling site in the present study, is a tributary of the River Thames, and passes close to several large centres of population in London (Wandsworth and the south west of the city), Carshalton and Croydon, both in Surrey. It provides a good example of a freshwater environment where there have been significant inputs of materials of man-made origin. The Wandle also accommodates a large population of sticklebacks with characteristic population densities in different sections of the river and varying degrees of parasitic infection, particularly with the cestode, *Schistocephalus solidus* (Chen, 1992).

Consideration has been made, in relation to environmental monitoring and fish health surveillance, of the use of the parasites of River Wandle sticklebacks as potential indicator species in this current research. Successive sections of this general introduction will deal with the biology and ecology of sticklebacks, general aspects of stickleback parasitology, environmental stress (principally pollution) and the prospects for research into parasites as environmental indicators (bioindices of disease, environmental change and pollution).

1.2. Biology of the stickleback (*Gasterosteus aculeatus*)

Several different species of stickleback are known to exist in European waters and the most common species of stickleback is the three-spined (*Gasterosteus aculeatus* L.). The other major species that exist are the four-spined stickleback (*Apeltes quadracus*), the nine-spined stickleback (*Pygosteus pungitius*). and the fifteen-spined stickleback (*Spinachia spinachia*). With the exception of the fifteen-spined stickleback which is a marine species, the others are normally found in freshwater environments. The three-spined stickleback, although found commonly in freshwater regions, is a euryhaline species and can also be located in brackish and estuarine environments (Wootton, 1976).

Identification and classification of the three-spined stickleback is made complex by the presence of several different forms, more commonly referred to as morphs. These morphs of the three-spined stickleback can be distinguished on the basis of the arrangement of the lateral plates (**Figure 1.1**). The three morphs of *Gasterosteus aculeatus* are the a) **trachurus**, b) **semiarmatus** and c) **leiurus** forms (Penczak, 1962).

1.2.1. Distribution of the three-spined stickleback (*Gasterosteus aculeatus*)

The three-spined stickleback can be found in most temperate and sub-polar regions. It is found between 35°N and 70°N in Europe, and in certain parts of Asia and North America. It is absent in tropical regions and there have been no reports of members of the family Gasterostidae in Africa or South America. In Europe, Asia and North America it is found in fresh, brackish and salt water, although restricted to the coastal waters of seas and oceans. A further restriction on its distribution is that it is not found in steep, fast-flowing streams and so is rare or absent in mountainous areas. The three-spined stickleback is most common in the slow-flowing backwaters and tributaries of rivers and in ditches, sheltered bays and harbours. It is also common in lakes and ponds (Bertin. 1925; Berg, 1949; and Munzing, 1959, 1963a).

Bertin (1925) has pointed out that the stickleback is most common in flat, low-lying areas such as eastern England, Holland and Belgium. The most noticeable feature of the distribution in Europe is that the trachurus form is restricted to the northern regions and the Black Sea, whereas the leiurus form occurs throughout western Europe but has a predominantly southern distribution **Figure 1.2**. The leiurus form is absent from the rivers of the Black Sea. Intergrades between trachurus and leiurus, the semiarmatus form, are found particularly in the North Sea and the western and north-western Baltic where the main distribution zones of leiurus and trachurus overlap.

Sticklebacks have shown considerable adaptation to differing environmental conditions. Certain populations of Balkan and Turkish sticklebacks which are found

in lakes of very high salinity (80-100 °/∞) have been described as having a substantial reduction in the number of lateral plates and exhibit many features which are dissimilar to sticklebacks occupying areas of lower salinity (Bacescu and Mayer, 1956; Munzing, 1963a).

In continental Europe, south of the English Channel, only the leiurus morph is resident in brackish and fresh waters. The southern part of the distribution limit for the leiurus morph extends from southern Spain to the south of Sardinia and through southern Italy to the south of Naples. Surprisingly, sticklebacks are entirely absent from Sicily but are present in the eastern side of the Adriatic coastline. Some of these southern sticklebacks lack lateral plates and have been classified as being a naked form of this species which was first recognised as hologymnura by Bertin (1925).

Bertin (1925) suggested that stickleback in Europe were genetically homogenous but that morphological variation was a result of difference in the environmental factors of salinity and temperature in which the fish developed. According to this theory, the leiurus form developed in fresh water at relatively high temperatures, while the trachurus form developed in salt water at relatively low temperatures and the semiarmatus form in areas intermediate in both salinity and temperature. It is difficult to defend this theory due to the distinct phenotype of the three forms which reflect distinct genotypes (Heuts, 1947a; Munzing, 1963a; Hagen, 1967). Heuts (1947a) showed that along the western coast of Europe, the sticklebacks can show two distinct patterns of lateral plates. In the extreme north only monomorphic trachurus populations which overwinter in the sea are present, but further south both high plated and low plated populations occur, the high plated populations overwintering in the sea, and the low plated populations remaining in fresh water (**Figure 1.2**).

In evolutionary terms the distribution of sticklebacks has been influenced by glacial activity, especially during the Pleistocene era (Munzing, 1963a; 1972). During the Riss-Wurm interglacial period, there were two distinctive geographical races. These being chiefly of the trachurus race which occupied a northern, circumpolar distribution, whilst the leiurus race had a more southerly distribution. Following the onset of the Wurm glaciation, the trachurus form migrated southwards and made

contact with several northerly elements of the leiurus race. The ability of the trachurus form to tolerate low water temperatures probably enabled it to survive periods of extremely depressed temperature relatively close to the ice-caps. With the Wurm - Riss glacial retreat, the range of the trachurus form expanded. From the northern North Sea it extended into the western Baltic and the southern North Sea as these areas became submerged. After submergence of the land connection between Britain and Europe, with the increasing warmth of the more northerly regions, the leiurus populations moved northwards, coming into contact with the trachurus form in the area of the southern North Sea and western Baltic. This meeting gave rise to the intergradation that resulted in the semiarmatus form providing an important component of stickleback populations in this area of western Europe.

1.3. Pathology and epidemiology of disease in stickleback

1.3.1. General observations on fish disease

Fish disease has been recognised as a problem of considerable magnitude. Increasingly, with the advent of modern intensive aquacultural practice, the need for screening and effective chemotherapy on control of bacterial, viral and parasitic diseases has been heightened. A general understanding of the epidemiology and biology of fish diseases in both feral and cultured fish is increasingly important.

One of the most profound stressors is pollution. Sinderman (1979) has considered in detail the effect of pollution upon disease in fish and shellfish. Sinderman's work has identified the seven major categories of disease/environmental contamination interactions. These are listed below, but it should be noted that in several instances the differences between the categories are rather arbitrary and incomplete.

a) Disease related to contaminant and pathogen stress such as fin erosion which is a disease of fish from polluted waters. Several authors have postulated that fin erosion in flatfish may be initiated by direct contact of tissues with contaminated sediments (Murchelano, 1975; Levin *et al,* 1972. Mearns and Sherwood (1974) and Sherwood and Mearns (1977), for example, suggested that toxic substances (sulfides, heavy

metals, chlorinated hydrocarbons, etc.) could remove or modify the protective mucus coat and expose epithelial tissues to the chemicals. Sherwood and Bendele (1975) reported that Dover sole from the California coast with severe fin erosion produced much less mucus than normal fish.

b) Stress-provoked latent infections. Some of the microbial diseases of fish have been shown to be provoked into patency by environmental stress (Wedemeyer, 1970; Snieszko, 1974). This seems to be true for kidney disease and furunculosis of salmonids, which often exist in carrier or latent states that can develop into active infections if fish are stressed.

c) Environmentally-induced abnormalities such as tumours. Lucke and Schlumberger (1941) were able to demonstrate the formation of tumour on the lips and mouth of the catfish (*Ameiurus nebulosus*) which had been exposed to organic toxicants. In flatfish, principally sole (*Solea solea*), Cooper and Keller (1969) reported that 12% of nearly 16,000 English sole had environmentally - related epidermal papillomas, with as many as 33 tumours per fish. Later, Sindermann (1976) found that mullet (*Mugil cephalus*) from Biscayne Bay (sampled from 1969-1970) possessed numerous wart-like fibromas which were classically associated with polluted environments.

d) Genetic abnormalities associated with mutagenic and tetratogenic agents (for instance organochlorine pesticides) (Huberman, 1975; Longwell, 1975).

e) Experimentally-induced lesions (experimentally-induced neoplasia and hypertrophy related to laboratory and field exposure to toxicants) (Ribelin and Migaki, 1975). Couch, 1975 reported the histopathological effects of the pesticides and related chemicals on the livers of fishes.

f) Contaminant effects on immune response and resistance to infection (immunosuppresion, lymphocystis) (Kolomiitseva *et al.*, 1969; Hemphill *et al.*, 1971; Khan and Hill, 1971; Jones *et al*, 1971; Koller, 1973; Street and Sharma, 1975, and

g) Pollutant-parasite interactions (changes in incidence of parasitism subsequent to exposure to xenobiotics). Boyce and Yamada (1977) found in laboratory experiments that sockeye salmon, *Oncorhynchus nerka*, smolts with preexisting parasitisation by the intestinal pseudophyllidean cestode *Eubothrium salvelini* were more susceptible to zinc poisoning than unparasitised siblings. Similarly, Pascoe and Cram (1977) found that survival times of the three-spined stickleback, *Gasterosteus aculeatus*, exposed to various concentrations of cadmium, were much shortened if the fish were parasitised by the larval cestode *Schistocephalus solidus.*

Each of the seven categories, based on the observation of Sindermann (1979), are potentially of use in assessing disease-pollution interactions in fish. In this investigation, the last category, that of pollution-parasite interactions has been considered.

Somewhat surprisingly, in a recent list of physiological, behavioural and biochemical variables that can be used as indicators of sub - lethal toxicity in fish, parasitism does not feature as a biological index (Abel, 1989). This either reflects a lack in ecotoxicological analysis or a problem with interpretation of the significance of parasitism as a biological indicator. Poulin (1992) described parasitism as being an excellent indicator and infers that there is a firm rationale for the greater establishment and consolidation of parasitism in environmental monitoring.

There are several ways in which toxic pollution can affect parasite infections in fish (**Figure 1.3**.). On one hand, the effects on the fish itself, such as immundepression (O'Neill, 1981; Zeeman and Brindly, 1981; Fries, 1986) could lead to greater parasite acquisition. On the other hand, toxic substances can negatively affect the parasites, either directly, for instance by harming the free - living stages, (Asch and Dresden, 1977; Evans, 1982a and b) or indirectly, by reducing the populations of invertebrate intermediate hosts (Guth *et al* 1977; Mance, 1987; Riggs and Esch, 1987; Riggs *et al* 1987; Brown and Pascoe, 1989). The ecological relevance of small changes in the prevalence or abundance of fish parasites is often easier to assess than that of small changes in term of overall toxicology. Therefore, monitoring changes in the

Any response, either negative or positive, in relation to the degree of parasitism of fish can be gainfully employed in environmental assessment. Several reports, including these of, Thulin, 1983; Riggs and Esch, 1987; Riggs *et al*, 1987; Valtonen *et al*, 1987 and Koskivaara *et al*, 1991 have assessed the relationships between parasitism, pollution and other environmental variables in several freshwater species and they have concluded that negative and positive changes in parasite infection, in fish, can be useful in freshwater environmental monitoring. This current investigation has tried to use parasitic indices as part of an assessment of the status of fish populations in an urban river environment.

1.3.2. The major parasitic infections associated with three-spined sticklebacks (*Gasterosteus aculeatus*)

Endo- and ecto-parasites are abundant in and on the three-spined stickleback, and six phyla, represented by ninety species of parasites have been identified for the three-spined stickleback (Bykhovskaya-Pavlovskaya *et al,* 1964; Chappell and Owen, 1969; Kennedy, 1974). **Table 1.1.** summarises the major parasitic infections identified as being present in three - spined sticklebacks.

Table 1.1. Major groups, sources of identification and distribution of parasites of the stickleback.

a) Protozoa

1. FLAGELLATA

Cryptobia branchialis (Chen, 1956). Gill ectoparasite. USSR.
Costia necatrix (Henneguy, 1884). Gill ectoparasite. Causative agent of costiasis. Northern USA.

Hexamita salmonis (Moore, 1923). Intestinal endoparasite. Northern USA.

2. SPOROZOA

Eimeria gasterostei (Thelohan, 1890). Liver endoparasite. USSR.

Myxidium gasterostei (Noble, 1943). Gall bladder endoparasite. USSR. Northern USA.

Sphaerospora elegans (Thelohan, 1892). Kidney tubule and urinary bladder parasite. USSR. UK. Northern USA.
Myxobilatus gasterostei (Parisi, 1912). Kidney tubule and urinary bladder parasite. USSR. Northern USA.
Myxobilatus medius (Thelohan, 1892). Kidney tubule endoparasite. USSR.
Glugea anomala (Moniez, 1887). Connective tissue endoparasite. USSR. UK. Northern USA.
Glugea pseudotumefaciens (Moniez, 1887). Ocular connective tissue endoparasite. USSR. UK. Northern USA.
Dermocystidium gasterostei (Elkan, 1962). Skin endoparasite. UK. *Henneguya sp.* UK.
Ceratomyxa sp. Intestinal endoparasite. Northern USA.
Thelohania baueri. Ovular parasite. USSR.

3. CILIATA

Hemiophrys branchiarum (Weinrich, 1925). Gill and skin ectoparasite. USSR.
Trichodina (*Cyclochaeta*) *gracillis* (Polyanskii, 1955). Gill and skin ectoparasite. Causative agent of Cyclochaetiasis USSR.
Trichodina (*Cyclochaeta*) *domerguei* (Wallengren, 1897). Gill and skin ectoparasite. USSR. UK. Northern USA.
Trichodina (*Cyclochaeta*) *reticulata* (= *Trichodina megamicroreticulata*) (Hirschmann and Partsch, 1955). Gill, skin and fin-ray ectoparasite. UK.
Trichodina (*Cyclochaeta*) *tenuidens* (Faure-Fremiet, 1943). Non-specific ectoparasite. UK.
Ichthyophthirius multifiliis (Fouquet, 1876), Skin endoparasite. White-spot disease. USSR. UK. Northern USA.

Trichophrya intermedia (Prost 1952) Gills ectoparasites USSR.
Glossatella amoebae. (Grenfell, 1887). Gill and skin ectoparasite. USSR.
Apiosoma (= *Glossatella*) sp. Skin ectoparasite. Northern USA.
Epistylis lwoffi (Faure-Fremiet, 1943). Skin and gill ectoparasite associated with Apiosoma. North America.

b) Platyhelminthes

1. MONOGENEA (All ectoparasite)

Gyrodactylus arcuatus (Bykhovskii, 1933). Gill and fin parasite. UK. USSR. Northern USA.
G. bychowskyi (Sproston, 1946). Gill parasite. USSR.
G. rarus (Wegener, 1909). Fin parasite. USSR. UK.
G. elegans (Nordmann, 1832). Gill and fin parasite. UK.
G. pungitii (Malmberg, 1956). UK.
G. alexanderi (Mizelle and Kritsky, 1967). Skin parasite. Northern USA.
G. avalonia (Hanek and Threlfall, 1969). Gill parasite. Northern USA.

G. canadensis (Hanek and Threlfall, 1969). Gill parasite. Northern USA.
G. lairdi (Hanek and Threlfall, 1969). Fin parasite. Northern USA.
G. memorialis (Hanek and Threlfall, 1969). Fin parasite. Northern USA.
G. terranovae (Hanek and Threlfall, 1969). Fin parasite. Northern USA.

2. DIGENEA

Brachyphallus crenatus (Rudolphi, 1802). Alimentary canal endoparasite. USSR. Northern USA.
Lecithaster gibbosus (Rudolphi, 1802). Intestinal endoparasite. USSR, Northern USA.
Lecithaster salmonis (Yamaguti, 1934). Intestinal endoparasite. Northern USA.
Podocotyle atomon (Rudolphi, 1802). Intestinal endoparasite. predominantly a marine parasite species. USSR.
Podocotyle reflexa (Creplin, 1925). Intestinal endoparasite. Predominantly a marine parasite species. USSR.
Cotylurus pileatus (Rudolphi, 1802). Swim-bladder parasite of which the sticklebacks acts as the intermediate host. definitive host for Cotylurus sp. is the seagull (Larus spp.), USSR.
Diplostomum spathaceum (Rudolphi, 1819). Parasite of th lens capsule of the eye. Stickleback is the intermediate host, the definitive host being a bird. *Diplostomum* spp. is the causative agents of "black-spot" disease. UK. USSR. Northern USA.
Diplostomum gasterostei (Williams, 1966). Endparasite of the pigment layer of stickleback eye. Sticklebacks are the intermediate host, birds being the definitive host. UK.
Diplostomum scudderi (Olivier). Endoparasite of the eye between the retina and the choroid. Sicklebacks are the intermediate host, birds are the definitive host. Northern USA.
Bunodera luciopercae (Muller, 1776). Intesyinal endoparasite. UK.
Bunodera mediovitellata (Zimbaluk and Roytman). Intestinal endoparasite. Northern USA.
Crepidostomum farionis (Muller, 1784). Intestinal endoparasite. Northern USA.
Crepidostomum cooperi (Hopkins, 1931). endoparasite (gut). Northern USA.
Crepidostomum spp. UK.
Phyllodistomum folium (Olfers, 1816). Urinary bladder endoparasite. UK.
Derogenes viricus (Muller, 1784). Alimentary canal endoparasite. Northern USA.
Nanophyetus salmincola (Chapin, 1926). Stickleback acte as an intermediate host for this parasite for which the definitive host is a carnivorous organism. Northern USA.
Posthodipolostomum minimum (MacCallum, 1926). Stickleback is the intermediate host, a bird is the definitive host. Northern USA.
Posthodipolostomum cuticola (Nordmann, 1832). Skin and fin endoparasite. Sticklebacks are the intermediate hosts, herons are the definitive hosts. USSR.
Tylodelphys clavata (= Diplostomum clavata) (Nordmann, 1832). Endoparasite of the humour of the eye. Stickleback acts as the intermediate host. UK.
Apatemon gracilis (Rudolphi, 1802). Intestinal endoparasite. Sticklebacks act as intermediate hosts, definitive host is a bird. Northern USA.

3. CESTODA

Triaenophorus nodulosus (Pallas, 1781). Encysted plerocercoids of this parasite are found in the liver of sticklebacks. Stickleback is the intermediate host, a piscivorous bird is the definitive host. USSR.
Bothriocephalus scorpii (Muller, 1776). Endoparasite. Sticklebacks act as intermediate hosts, the definitive host is a piscivorous marine fish species.
Bothriocephalus claviceps (Goeze, 1782). Endoparasite. UK. Northern USA.
Diphyllobothrium dendriticum (Nitzsch, 1824). Endoparasite of the body cavity and viscera present as the plerocercoid larval stages. Stickleback acts as an intermediate host, gulls and terns are the definitive hosts. USSR. UK.
Diphyllobothrium norvegicum (Vik, 1957). Encysted plerocercoids of this endoparasite are present in the stomach, intestine and liver. Sticklebacks are one of the intermediate hosts for *D. norvegicum*, the other intermediate hosts being the char (*Salvelinus alpinus* and *S. fontinalis*) and trout (*Salmo trutta*). Definitive hosts for theis tapeworm include, cats birds and humans. UK. Northern USA.
Schistocephalus solidus (Muller 1776). Endoparasite, with plerocercoids being found in the peritoneal cavity. Sticklebacka are the intermediate hosts, with piscivorous birds being the definitive hosts. USSR. UK. Northern USA.
Proteocephalus cernuae (Gmelin, 1790). Intestinal endoparasite. USSR. Norther USA.
Proteocephalus filicollis (Rudolphi, 1802). Intestinal endoparasite. USSR. UK.
Proteocephalus pungitensis (Hoff and Hoff, 1929). Endoparasite. Northern USA
Eubothrium crassum (Bloch, 1779). Intestinal endoparasite. UK.
Cythocephalus truncatus (Pallas, 1781). Intestinal endoparasite. Northern USA.

c) Aschelminthes

1. NEMATODA

Raphidascaris acus (Bloch, 1779). Endoparasite which exists as an encysted form in the liver, body cavity, intestinal walls and gonads. S ticklebacks act as intermediate hosts, definitive hosts being piscivorous birds. USSR. Northern USA.
Raphidascaris cristata (Linstow, 1872). Endoparasite. Stickleback is an intermediate host. UK.
Cystidicola farionis (Fischer, 1798). Swim-bladder endoparasite. USSR. Northern USA.
Camallanus lucustris (Zoega, 1776). Intestinal endoparasite. USSR.
Camallanus truncatus (Rudolphi, 1814). Intestinal endoparasite. USSR.
Eustrongylides sp. Endoparasite of the musculature and body cavity. Sticklebacks are the intermediate hosts, birds are the definitive hosts. Northern USA.

2. ACANTHOCEPHALA

Neoechinorhynchus rutili (Muller, 1780). Intestinal endoparasite. USSR. UK. Northern USA.
Pseudoechinorhynchus clavula = (*Acanthocephalus clavula* ? *Echinorhynchus clavula*) (Dujardin, 1845). Intestinal endoparasite. USSR. UK.

Metechinorhynchus salmonis (Muller, 1780). Intestinal endoparasite USSR.
Acanthocephalus lucii (Muller, 1776). Intestinal endoparasite. USSR. Northern USA.
Pomphorhynchus laevis (Muller, 1776). Intestinal endoparasite. USSR. UK.
Corynosoma semerme (Forssell, 1904), Endoparasite. Sticklebacks are the intermediate hosts, with marine mammals and piscivorous birds being the definitive hosts. USSR.
Corynosoma strumosum (Rudolphi, 1802). Coelom, muscle and internal organ endoparasite. Sticklebacks are the intermediate hosts, the definitive hosts are marine mammals and birds. USSR.

d) Annelida

1. HIRUNDINEA (Leeches)

Piscicola geometra (Linneaus, 1761). Endoparasite. USSR.

e) Arthropoda

1. CRUSTACEA

i) **Copepoda**

Ergasilus auritus (Markevich, 1940). Gill and fin ectoparasite. USSR, Northern USA.
Ergasilus turgidus (Fraser, 1920). ectoparasite. Northern USA.

Thersitina gasterostei (Pagenstechner, 1861). Opercular ectoparasite. Northern USA,
Caligus lacustris (Steenstrup and Lutken, 1861). Internal opercular ectoparasite. USSR. UK. Northern USA.
Caligus clemensi (Parker and Margolis). Fin ectoparasite. Northern USA.
Lernaea esocina (Burnmeister, 1833). Skin and gill ectoparasite. USSR.
Lernaea cyprinacea (Linnaeeus, 1758). Skin ectoparasite. UK.
Salmincola edwardsii. Ectoparasite. Northern USA.

ii) **Branchiura**

Argulus foliaceus (Linnaeus, 1758). Skin and gill ectoparasite. USSR UK.
Argulus canadensis (Wilson, 1916). Ectoparasite. Northern USA.

f) Mollusca

Unionidae gen. sp. Ectoparasite of gills. USSR. UK. Northern USA.

As indicated in **Table 1.1** the stickleback acts as host to a very wide variety of parasites. In many instances the stickleback acts as the definitive host, however, there are several notable examples, principally among the Digenea and Cestoda, where the

stickleback plays an important role as an intermediate host. The following sections describes the life cycle of one such cestode, *S. solidus*, which employs sticklebacks in this manner in the River Wandle.

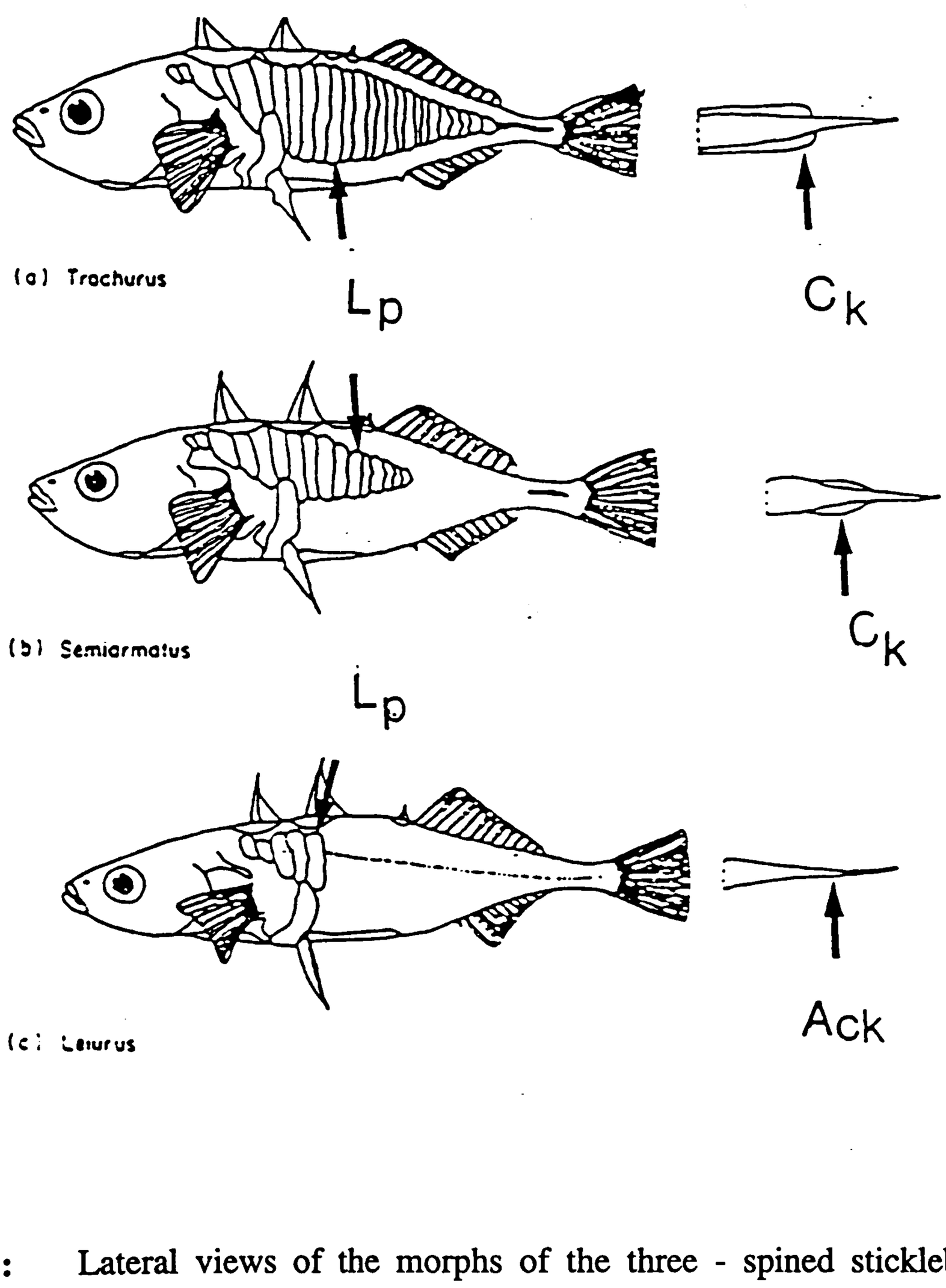


Figure 1.1: Lateral views of the morphs of the three - spined stickleback (*G. aculeatus*) (a) trachurus, (b) semiarmatus and (c) leiurus on the right hand side of each image is a diagram of the tail region viewed from the dorsal side to show the extent of lateral caudal keels (Lp = lateral plates; Ck = caudal keel; Ack = absent caudal keel) (Penczak, 1962).

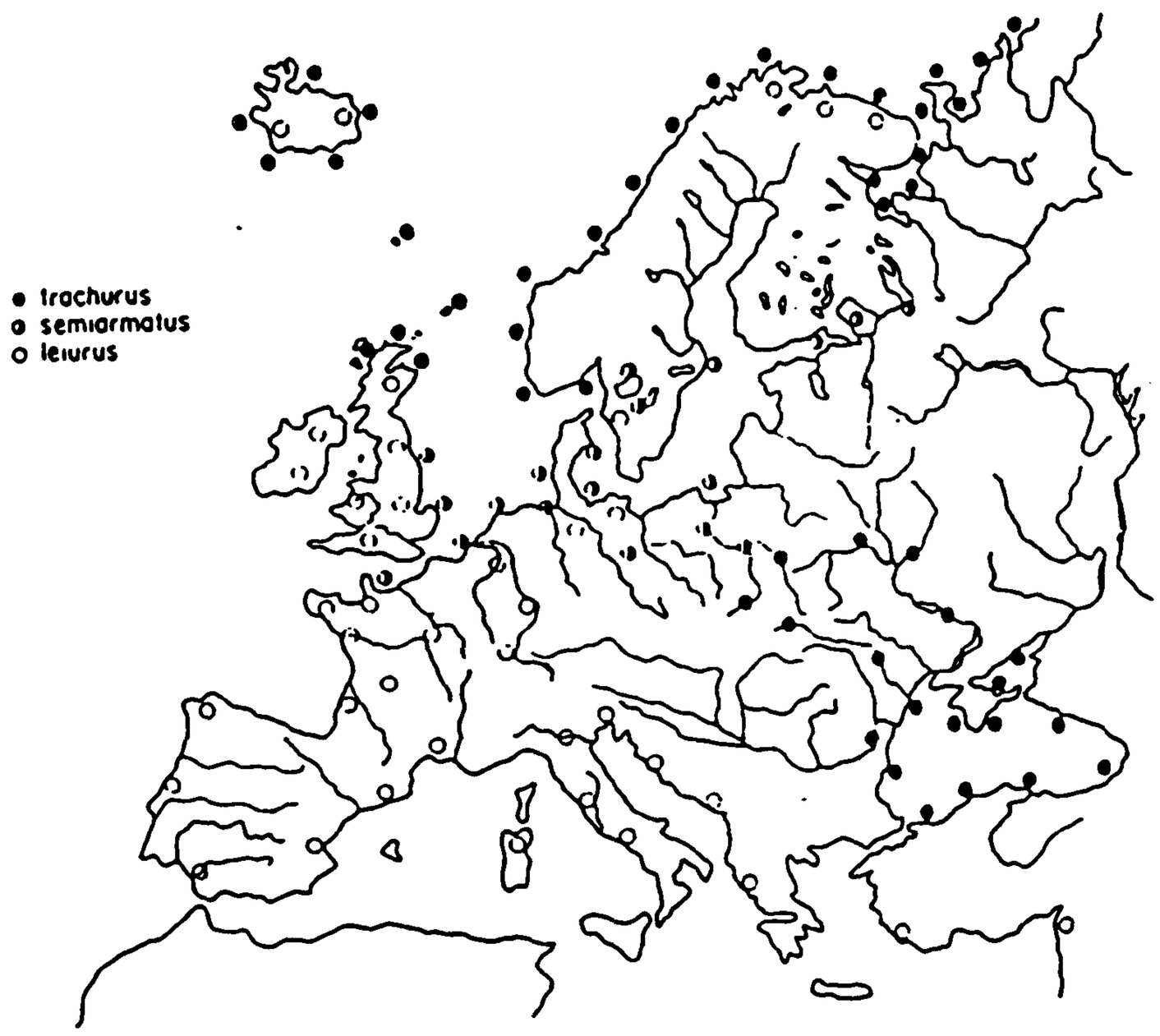


Figure 1.2: The distribution of the trachurus, semiarmatus and leiurus morphs of the three - spined stickleback (*G. aculeatus*) (Bertin, 1925).

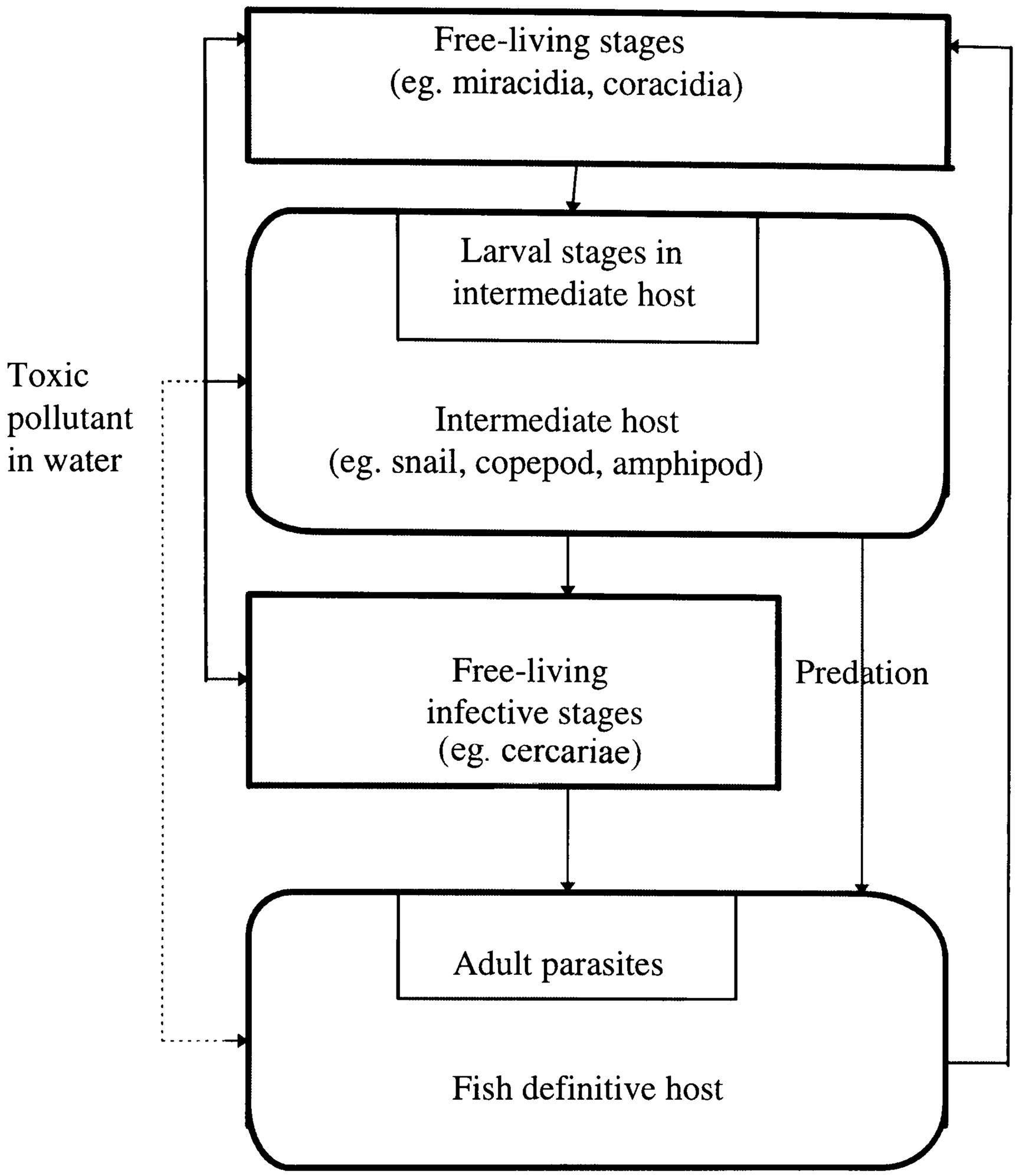


Figure 1.3: Speculative effects of a water-borne pollutant on the host-parasite system involving an intermediate host. (Solid lines denote effects which maybe beneficial for fish populations whereas the broken line represents a det rimental effect) (Figure adapted from Poulin, 1992).

1.3.3. The life-cycle of the pseudophyllidean cestode, *Schistocephalus solidus*

1.3.3.1. General comments on *Schistocephalus solidus*

S. *solidus* is a pseudophyllidean cestode and is characterised by possessing progenetic plerocercoids. Plerocercoids of *S. solidus* provide the basis for a number of very elegant ***in vitro*** parasite models (Smyth, 1990a).

Plerocercoids of *S. solidus* reach an advanced stage of progenesis in the fish host, typically sticklebacks (*G. aculeatus*) with genital anlagen or primordia (groups of cells in a larval stage which, when they multiply and develop will form a specific adult structure) very evident. Additionally, this parasite exhibits well developed proglottid formation. Plerocercoids undergo rapid maturation, typically in 36 hours, in the avian definitive host (Hopkins and Smyth, 1951; Orr and Hopkins, 1969a).

1.3.3.2. Host utilization of *S. solidus*

Most adults of *S. solidus* have been found to occur in avian hosts. Generally, *S. solidus* is non - specific in its choice of definitive hosts and has been found in a number of bird species, that include sticklebacks in their diet such as mallard (*Anas platyrhynchos*), heron (*Ardea cinerea*), black-headed gull (*Larus ridibundus*) and terns (*Sterna paradisea* and *S. hirundo*) (Owen, 1960; Creutz, 1963; Lemmetyinen, 1973).

It has been shown that *S. solidus* establishes well in ducks in experimental infection (*Anas platyrhynchos*) (over 50% establishment) (McCaig and Hopkins, 1963) and in chicks (*Gallus domestcus*) (40 - 50% establishment) (Orr and Hopkins, 1969a). Presumably, the ease of establishment of *S. solidus* is due to the progenetic nature of the plerocercoid which has sufficient endogenous energy substrates that enable it to achieve rapid maturation without having to depend upon exogenous reserves.

1.3.3.3. The life - cycle of *S. solidus*

The life-cycle of *S. solidus* is similar in many respects to that of other pseudophyllideans like *Diphyllobothrium dendriticum*. There are, however, some differences in rates of maturation and choice of definitive host between *S. solidus* and *D. dendriticum* (Clarke, 1954; Hopkins and Smyth, 1951).

There are several important developmental phases associated with the life-cycle of *S. solidus*. Each of these phases is marked by significant changes in the morphology of the larval stages of the parasite which enable it to penetrate new hosts and readily adapt to contrasting physiological conditions (i.e. gut pH, temperature, salinity, oxygen concentration). There is also the transition, during morphogenesis, from the free-swimming (and free-living) coracidial stage to the parasitic procercoid and plerocercoid stages.

Defaecating infected birds release *S. solidus* eggs, which have quinone-tanned external surfaces, and these can enter aqueous environments. Each egg develops into an embryonated form, the coracidium, at 26°C over a period of 8 days and in the presence of adequate light they hatch to release the free-swimming larva.

The free-swimming coracidium is ingested by the first intermediate host, *Cyclops* spp., in which it develops in the haemocoele to become a procercoid larva. Several species of *Cyclops* have been shown to be first intermediate hosts, including, *C. agilis*, *C. scutifer* and *C. abyssorum* (Kuhlow, 1953b). The infective procercoid reaches full maturity in *Cyclops* within 10 days (Orr and Hopkins, 1969a).

In turn, *Cyclops* are ingested by the second intermediate host, the teleost fish, the three-spined stickleback (*Gasterosteus aculeatus*). The procercoid then bores through the stickleback gut to the peritoneal cavity where it develops into the next stage, the plerocercoid. The plerocercoid exhibits considerable growth and rapid maturation in the peritoneal cavity of the stickleback.

Infected sticklebacks are then consumed by the definitive, typically avian, hosts such as gulls and ducks. The plerocercoid migrates to the gut wall where it rapidly develops into the mature cestode parasite. Maturation from the plerocercoid to the adult worm takes between 36-48 h. An outline description of the life-cycle is provided in **Figure 1.4**.

Key to Figure 1.4:

1.- Release of egg in avian faeces

2.- Development of embryonated egg

3.- Formation of the free-living stage-the coracidium

4.- Formation of the 1st intermediate stage-the procercoid (in *Cyclops* sp.)

5.- Formation of the 2nd intermediate stage-the plerocercoid (in the stickleback).

1.3.3.4. Pathogenic and behavioural effects of *S. solidus* infection on the three - spined sticklebacks

Levels of plerocercoid infection in stickleback populations vary greatly. Some, though, can exist at very high prevalence and intensity levels. Such infections can have significant effects on the behavioural and physiological responses of fish populations to exogenous stressors i.e. predator and pollution.

Ligula intestinalis, another cestode parasite, has been demonstrated to have profound effects on the sexual morphology and reproductive activity of sticklebacks. Kerr (1948) reported that infection with *L. intestinalis* led to castration of sticklebacks and hence infertility. *S. solidus*, however, does not cause castration but has been shown to effect other changes in sticklebacks.

Evidence indicates that the plerocercoid is a highly efficient converter of energy and can use substrates much more effectively than its host. Predictably, parasitism by the plerocercoid induces higher mortality amongst the host sticklebacks and reduces overall fish condition. (Walkey and Meakins, 1970; Milinski, 1984, 1990).

Behavioural changes related to feeding, respiration and host competition have also been studied in relation to parasitism in sticklebacks (Milinski, 1984, 1990; Walkey and Meakins, 1970; Tierney *et al*, 1996). Several studies have indicated that a major cause of mortality in plerocercoid - parasitised sticklebacks is asphyxiation.

Pathogenic effects of *S. solidus* infections were manifested in several organ systems of the stickleback's male and female populations (Tierney *et al*, 1996). Weight, as a function of length, was lower in infected sticklebacks compared with uninfected fish during autumn and spring. In winter and summer both categories of fish were in equally poor condition. In early autumn, the hepatosomatic indices of newly infected fish were higher than those of uninfected fish, perhaps due to a pathological response. Thereafter, relative liver sizes of uninfected and infected sticklebacks were comparable until spring, when they increased sharply in uninfected sticklebacks but remained stable at a low level in infected sticklebacks. Few infected fish reached maturity. The only males to attain maturity whilst sustaining an infection of *S. solidus* were in particularly good condition. Nuptial colouration, kidney hypertrophy and testes size were unimpaired in these mature infected males, but whether they were capable of successful reproduction remains debatable.

The effect on infection by *S. solidus* on prey size selection by sticklebacks has been described in varying ways in different studies. Ranta (1995) observed that infected fish ate slightly less but fed more selectively on large prey than non - infected fish. It follows that, in the short - term, the net energy gain in foraging is larger for infected fish. This matches the expectation that infected sticklebacks compensate energy losses due to *S. solidus* . Barber and Huntingford (1995), on the other hand, found that the presence of *S. solidus* plerocercoids in the body cavity constricts the stomach, increases the handling time for large prey and consequently reduces the profitability of such prey for infected fish. This is reflected in a switch in dietary preference from large to small prey in the laboratory and in altered stomach contents and impaired nutrient reserves in the sticklebacks.

This inconsistency is further underlined by the study of Wedekind *et al*, (1996) into the predator - prey interaction between laboratory bred three - spined sticklebacks, from two parasitised and one unparasitised population, and different prey types namely infected and uninfected copepods and size - matched *Daphnia* as alternative prey. Copepods with infective procercoids were more active, had a lower swiming ability and were easier to catch than uninfected controls. The sticklebacks preferred moving copepods. Parasitised copepods were preferentially attacked and consumed. Whether the stickleback's parent population was parasitised or not appeared not to influence the outcome of such experiments. The sticklebacks switched from *Daphnia* to (uninfected) copepods in the course of a hunting sequence, this switch occured in smaller fish. With this strategy the fish maximised their feeding rate, *Daphnia* were easier to catch than copepods but increasingly difficult to swallow when the stomach was filling up especially for smaller fish. However, there was no indication that sticklebacks from infected populations either consumed *Daphnia* rather than copepods or switched later in the hunting sequence to consuming copepods than fish from an uninfected populations. Thus, sticklebacks, did not avoid parasitised prey, although *S. solidus* usually has a high prevalence and caused a strong fitness reduction in its stickleback hosts.

Barber *et al*, (1995), have observed the effect of *S. solidus* infection on the shoaling behaviour of sticklebacks. When satiated, fish spent a greater proportion of time within one body length of a shoal of conspecifics and spent less time out of visual contact with the shoal than after periods of starvation. Complex effects were seen on this basic shoaling behaviour when fish were infected. When satiated, they spent less time than uninfected fish within one fish length of the shoal yet stayed within visual contact. When food deprived, there were no behaviour differences between infected and uninfected fish.

1.4. Notes on the sampling site investigated in this study: The River Wandle

London and its rivers have been of long-standing historical and biological interest. Some of the most profound changes in water quality have been observed over the past century and a half due to urbanisation and its associated pollution load and subsequent improvement of river water quality.

Fish population studies on the Wandle are not very extensive. Historical records show that the Wandle did once support a good fishery with evidence of several salmonid species having been present, including brown trout (*Salmo trutta*), rainbow trout (*Salmo gairdneri*, [now *Oncorhychus mykiss*]) and salmon (*Salmo salar*). Other species, including cyprinids, cottids and gobies were also reported as having been found in the Wandle during the 17th, 18th and early 19th centuries (Wheeler, 1958).

With the Industrial Revolution came the concomitant problem of pollution. During the period from the mid-1800's to the late 1960's there was a considerable decline in the number of fish species recorded in the Wandle. The decline in water quality in the Wandle, much of it associated with a heavy sewage load at Beddington, Wandle Valley and Wimbledon gave rise to the river becoming little more than an open sewer. Stickleback populations were abundant in the river but showed a significant decline during the late 1950's-1960's. Marlborough (1963) considered the occurrence and distribution of the stickleback, in this now highly polluted river, to be due to their tolerance of pollution. However, it would appear that there were limits even to the tolerance of the stickleback to pollution and from 1962-1969 there were no records of any fish in the Wandle (Marlborough, 1969).

Since the early 1960s, considerable progress has been made in cleaning up the Wandle. Several small old sewage works were closed and their flow diverted to the Crossness works. Industrial discharges now go to the sewage system rather than directly to the river. Beddington sewage works, which serves Croydon, has been rebuilt and extended and is now the main sewage effluent discharge.

There has never been a comprehensive investigation of parasitism in sticklebacks in the Wandle, and the provision of such as analysis is part of this current investigation. Sticklebacks, due to their widespread distribution in the Wandle (Chen, 1992), also form a valuable marker species. Through exploiting the River Wandle as a sampling site, in combination with the wide distribution of the stickleback, this study has been able to examine an interesting set of related issues namely, pollution (xenobiotics), parasitism and fish ecology.

(b): History and present environmental status

The River Wandle is formed by the conjunction of streams from various springs, the largest of these being found at Carshalton and Croydon. The abundant clear chalk water in the river which once supported a good fishery, including trout, attracted industry to the Wandle Valley. Industrial premises grew up along the banks during the 19th century based on power from the water - wheel.

A recent report from the Environment Agency (Environment Agency, 1996) has described the history and present environmental status of the River Wandle. By the mid 1930's the quality of the water was very poor for three main reasons. With the increase in the population and water usage, heavy demands were made on potable supplies from groundwater and this reduced the natural springs which fed the river. Secondly, this led to an increase in the quantity of sewage effluent discharged to the river from three sewage works, Beddington, which served Croydon, Wandle Valley, which served Mitcham and Merton and the Wimbledon works. All of these allowed partially treated sewage to enter the river which, at that time, accounted for over 90% of the total average flow. Finally, industrial discharges contributed to the general decline of river quality.

In 1967 the Sutton and District Water Company increased the abstraction of groundwater for potable supply near the springs at Carshalton Ponds. It was considered that this increased abstraction would cause the ponds to dry up at times and the Water Company agreed to maintain the flow in the Carshalton Ponds from

Goat Bridge, just upstream of the confluence with the effluent from Beddington works. These ponds look rather turbid during summer months due to low flows from the springs feeding the lake and the fact that some chalk particles are suspended in the water.

By the end of the 1960's implementation of the Rivers (Prevention of Pollution) Acts had reduced some industrial pollution, and a start had been made on the major capital work required to improve the quality of the sewage effluents. The quality of the Wandle downstream of the sewage works was still poor.

Comissioning of the new activated sludge works at Beddington in 1970 and the diversion a year later of the entire flow of Wimbledon works and a part of that from Wandle Valley to Crossness works gave rise to an improved water quality, although the effect on biological life was not immediate. Biodiversity levels in the river have been stable since 1973. The river now supports large populations of a limited number of species including the water louse, *Asellus aquaticus*, certain molluscs, leeches, midge larvae and oligochaete worms.

Extensions to Beddington sewage works came into operation early in 1978, and the quality of the lower reaches of the river is now high enough to support a coarse fishery where physical features are also suitable. Unfortunately spillages of chemicals from the large industrial area draining to this river have resulted in fish mortalities on occasions.

The current consent standard for this industrial area is currently under review and it is planned to install fine screens on the effluent to prevent plastic material reaching the river in the near future. The River Wandle's main tributary, the River Graveney is confined within a concrete channel for its entire length from the source near Selhurst to its confluence with the River Wandle at Colliers Wood. This river, as well as the Wandle, drains a largely impervious urbanised area so that during periods of rainfall large amounts of road runoff discharge into the river causing the quality of the river water to deteriorate and the flow to increase rapidly.

In the mid seventies the River Graveney suffered from chronic oil pollution, the source being the Selhurst Railway Depot. Drainage works were undertaken by British Rail and a new oil interceptor was installed. In 1986, whilst demolition work was being carried out at a disused factory, oil escaped into the drainage system from old oil storage tanks and in 1987 some cable oil was lost into the track drainage system at Selhurst Railway Depot. In 1995 the Wandle was turned a purple colour and the culprit, an ink company based at Earlsfield, were fined £400. The river suffered from oil pollution again early in 1996 following vaddalism at a building contractor's depot.

Table 1.2 summaries chemical quality data for a number of sites on the River Wandle collected in 1995 by the Environment Agency. **Table 1.3** described heavy metal values at a single Wandle site in 1995.

Table 1.2: Chemical quality readings from sites on the River Wandle. Results are expressed in mg/l and are the mean of samples taken during 1995 (Environment Agency Report 1995).

Location	pH	Sus.	B.O.D	D.O.	NH3	NO3	CL
Goat Bridge u/s Beddington STW	7.93	6.90	0.47	92.60	0.07	6.89	29.84
Plough Lane Wimbledon	7.66	8.00	2.03	59.79	3.25	10.61	63.00
The Causeway Wandsworth	7.76	9.84	2.52	75.50	6.69	9.94	63.66

Table 1.3: Heavy metals (µg/l) measured at the Causeway Wandsworth by the Environment Agency (1995).

Chromium	Zinc	Nickel	Copper	Cadmium	Lead	Mercury
1.25	32.25	0.75	6.78	10.16	12.85	0.04

The principal effects on the quality of the rivers are caused by treated sewage effluent, storm overflows from the foul sewer system, untraced wrong connections to the

extensive surface water system, run - off from paved areas in times of storm and spillages of chemicals and oil at factories and on highways.

(c): River Quality Assessment

In June 1994 the new General Quality Assessment Scheme (GQA) was introduced for the periodic assessment of freshwater quality. The scheme aims to provide a means to measure water quality on a nationally consistent level without any relation to the use of the stretch and is only used to assign the most likely class for a river for a given time period. It is not used to assess a compliance with a water quality objectives.

The GQA scheme for freshwater will assess both the chemical and biological quality alongside each other. At a later stage a nutrient and aesthetics component to the scheme may also be introduced.

The GQA scheme will run in parallel with Statutory Water Quality Objectives Use Classes (SWQOs). The River Ecosystems Use of the SWQO scheme has been introduced by the Surface Water (River Escosystems) (Classifications) Regulations 1994, but the use - related standards will not apply until formally implemented by regulations for individual catchments. Until SWQOs are fully introduced existing RQOs will be translated into the corresponding River Ecosystem use classess. These derived objectives will be informal and non statutory.

(d): Chemical Components of the General Quality Assessment Scheme and Monitoring

The GQA scheme is based upon three determinants: BOD, total ammonia and dissolved oxygen and the grade limits are summarised in **Table 1.4.** It should be noted that the GQA uses 3 year's worth of data; the use of a larger data set reduced the possibility of incorrectly grading a stretch to 25%. Using this grading system the most recent grading of the River Wandle is E (poor).

Table 1.4: Chemical grades of the GQA Assessment for Rivers and Canals (1994).

Grade	Dissolved oxygen (% saturation) 10%	BOD (ATU) mg/l 90%	Total ammonia as N (mg/l) 90%
A	80	2.5	0.25
B	70	4.0	0.60
C	60	6.0	1.30
D	50	8.0	2.50
E	20	15.0	9.00
F	20	-	-

1.5. Toxicity and metabolism of xenobiotics in fishes

Fish studies have formed a significant basis for the evaluation of freshwater and marine environmental contamination. Both wild and cultured fish species have been used in toxicological investigations. Classically, most fish toxicological assessments have focused upon acute toxicity, the derivation of quantitative modelling techniques, bioaccumulation studies and biotransformation (metabolism, both ***in vivo*** and ***in vitro***).

Some of the earliest demonstrations of the use of fish in extensive toxicological assessment pre-date 1950s, with Goodnight (1942) demonstrated the usefulness of cyprinids, mainly goldfish (*Carassius auratus*), in evaluating the toxicity of phenols. Since then the range of species used has broadened considerably. Classical regulatory species used in toxicity testing, include, sheepshead minnow (*Cyprinodon variegatus*), fathead minnow (*Pimphales promelas*), the Japanes medaka (*Oryzias latipes*) and the zebrafish (*Brachydanio rerio*) (Saarikoski and Viluksela, 1982; Geiger *et al*, 1986).

Phenolic wastes, among the various toxicants which are of particular interest to this study, affect aquatic life ecosystems by their direct toxicity to fish and other aquatic life. These distrupt community structure and trophic relationships and even the flesh of fish can be tainted especially in the case of chlorinated phenols. When phenols are subjected to oxidation reactions they can also contribute to the depletion of the dissolved oxygen concentration of polluted waters; these reactions can therefore make it difficult to assess the direct importance of phenols as pollutants simply from examination of toxicity data (Alabaster and Lloyd, 1980).

Aquatic species are constantly in danger of being exposed to a great diversity of foreign compounds, in that chemicals released into the atmosphere or on land by the activities of man frequently find their way into tracts of water, in addition to those entering directly in effluents. Toxicity evaluation has been usually detemined in non - diseased states, i.e in healthy organisms, and thus is not wholly representative of real populations where other parameters, such as bacterial and parasitic disease, may have an influence on the effect of a toxicant.

Another major area of investigation in aquatic toxicology has been biotransformation. Biotransformation in vertebrates, broadly speaking, refers to the hepatic metabolism of xenobiotics through phase I (oxidation, reduction and hydrolysis) and phase II (conjugation) biochemical metabolic processes. In vertebrates, foreign organic compounds undergo a wide variety of biochemical transformations to allow for their more rapid elimination in water - soluble forms. These biotransformations include: conjugation, nitro - and azo - reduction and ester and ether cleavage. Although there are oxidations and reductions catalyzed by enzymes that are non - hepatic and non - microsomal, the major metabolic transformations occur in the liver and are specifically associated with the endoplasmic reticulum. Several extensive reviews exist on biotransformation in aquatic organisms, both vertebrates and invertebrates, including those of (Chambers and Yarbrough, (1976), Lee, (1981) and Livingstone, (1985).

The enzyme systems associated with biotransformation are present in almost all vertebrate groups and have been best documented in mammals (Chambers and Yarbrough, 1976; Lee, 1981 and Livingstone, 1985). It was generally accepted in the past that fishes process lipid soluble organic substances by the rapid elimination of these substances into the external aqueous environment through the activity of the gills. However, it was later demonstrated that some fish species could detoxify xenobiotics and that the toxicity of several xenobiotics could be related to hepatic metabolic activity (Buhler, 1966; Lech, 1974). Further, it appeared that the gill in general is a rather poor excretory unit, whereas the kidneys of fishes are capable of active excretion of many biotransformed derivatives of toxicants (Adamson, 1967).

Little is known about the influence of disease on the metabolic processes of detoxification of xenobiotics in fish. There have, though, been a number of investigations on the effect of liver neoplasia on the metabolism of the polyaromatic hydrocarbons such as aminofluorene, anthracene, 3,4 benxopyrene and coronene, in fishes (Collier *et al*, 1986; Collier and Varanasi, 1988; Smith *et al*, 1993; Myers *et al*, 1998). The effect of parasitism on biotransformation, is however, a relatively unworked area of investigation.

This aspect of parasitic infestaion and its possible role in influencing the biotransformation of phenol in sticklebacks, a chosen aquatic monitor species, is a concern of this study. Phenols mainly undergo phase II reactions and these are commonly glucuronic acid and sulphate conjugations. These reactions are also known to occur in fish. The formation of glucuronides is an important pathway in the conjugation of foreign compounds in animals. Glucuronides are excreted mainly into the bile, hence this study concentrated on bile fluid in the recovery of the biotransformed products of phenols.

A latter part of the present study has been devoted to assessing through preliminary laboratory experimentation, the effect of a parasite, *S. solidus*, on the toxicity and metabolism of phenol and pentachlorophenol (PCP) in the stickleback (*G. aculeatus*).

This work has further been consolidated by the use of specific inhibitors that have provided a further insight into the role of metabolism on the toxicity of phenol and PCP in diseased and non - diseased states.

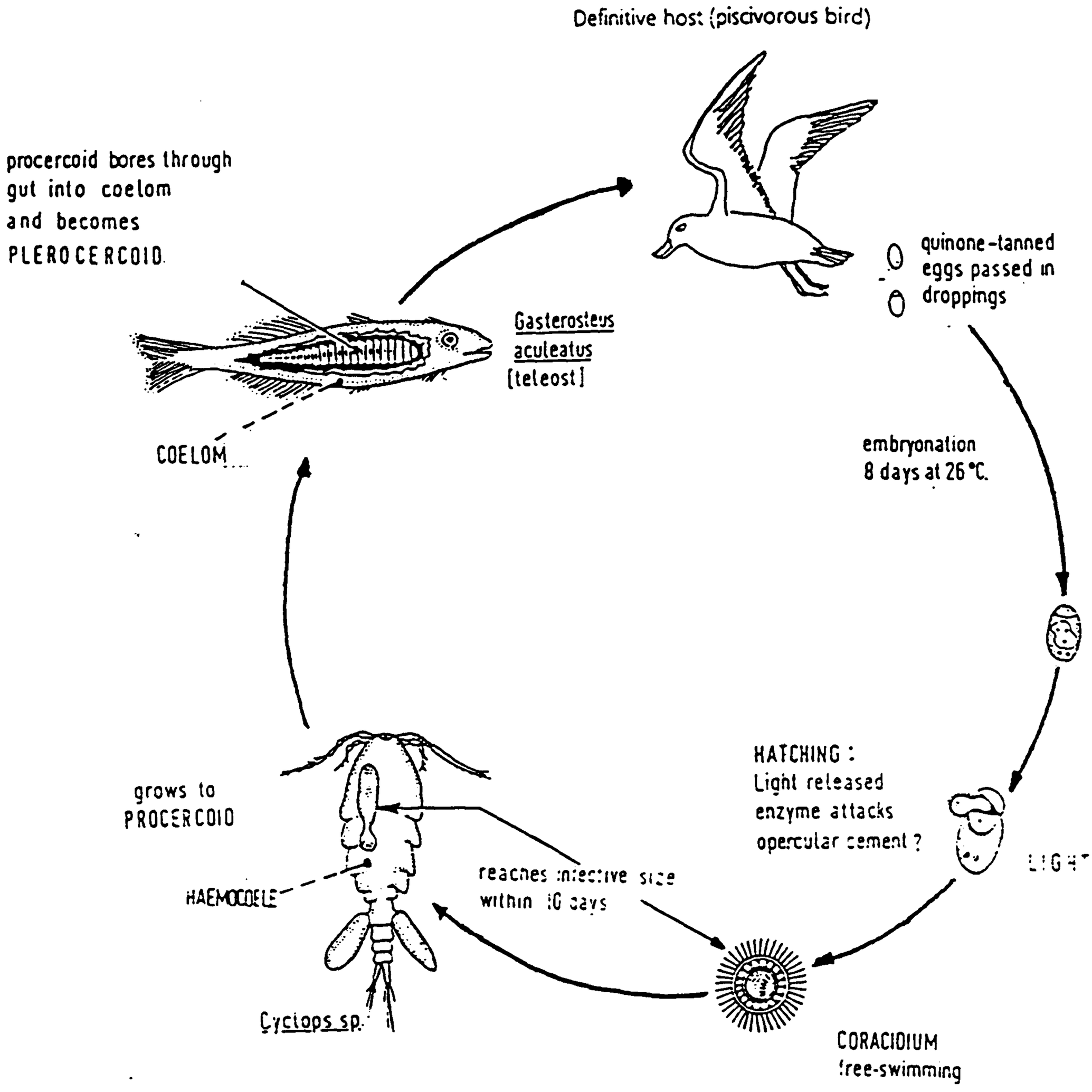


Figure 1.4: The life-cycle of *Schistocephalus solidus*. (From Smyth, 1994).

1.6. Studies on the *in vitro* biotransformation of phenol in a freshwater fish, perch (*Perca fluviatilis*), in relation to chronic hepatic disease caused by infection with the hepatic cestode, *Triaenophorus nodulosus*

1.6.1. *Triaenophorus nodulosus* infection in perch (*Perca fluviatilis*)

Additional studies undertaken during the progress of this research, alongside the work on sticklebacks, have examined the influence of parasitic disease on biotransformation, using perch (*Perca fluviatilis*) as a test organism in relation to infection by *T. nodulosus*. Unlike *S. solidus*, the cestode, *T. nodulosus*, is a hepatic parasite which utilises the perch as an intermediate host (the definitive host for *T. nodulosus* being the pike, *Esox lucius*).

On the assumption that plerocercoids of *T. nodulosus* in the liver have direct effect on the metabolic processes of detoxification, a series of ***in vitro*** investigations were set up to evaluate this hypothesis. Previous investigations, notably Smith *et al*, 1993, clearly demonstrated that in diseased fish (affected by neoplastic lesions and hepatic necrosis) there was reduced excretion of glucuronide conjugates with their concomitant accumulation in the gall bladder. The principal aim of this aspect of the research programme was to ascertain, through implementation of short - term experimental protocols, if hepatic parasite infection and the formation of liver lesions had any significant bearing on the biotransformation of phenol. It must be emphasised that these investigations were of a preliminary nature. They attempted to determine the presence or absence of such phenomena rather than attempting to measure their magnitude.

Additionally, a further aim of this research, was to evaluate if there was any benefit in developing the *T. nodulosus* infection as a model for determining the detailed relationship between the histopathology of parasitic disease and changes in biotransformation metabolism. This was to be achieved by considering metabolic changes in concert with the progression of the gross pathological changes induced in perch liver by *T. nodulosus*.

1.6.2. The morphology and life-cycle of *Triaenophorus nodulosus*

The study of the genus *Triaenophorus* began more than 200 years ago. Early reports described the cestode as long worms from the intestine of pike and the liver of perch (Hartmann, 1688, cited by Scheuring, 1930). There are several species of the genus *Triaenophorus*, listed in Kuperman (1973) such as *T. nodulosus* which was described by Pallas (1781), also *T. crassus* was described by Forel (1868) and *T. stizostedionis* described by Miller (1945c).

The sexually mature specimens of *Triaenophorus* collected from the intestine of pike (*Esox lucius*), which is the definitive host, are elongate, tapeworm-like in form without any external segmentation. At the anterior end exists the attachment organs / apparatus which consists of a scolex with two pseudobothria and two pairs of hooks. Between the species of *Triaenophorus* there are differences in the structure of the attachment hooks and these variations are the basis of identification of each species of the parasite (Hjortland, 1928; Miller, 1952; Dubinina, 1964; and Kuperman, 1965).

The life - cycles of tapeworms of the genus *Triaenophorus* are quite complex and involve two intermediate and one definitive aquatic host. The hermaphroditic sexually mature generation of the parasite lives in the intestine of the definitive host. Ciliated larvae - coracidia - develop in the eggs of the worms and then hatch. Coracidia floating and swimming in the water are ingested by copepods, their first intermediate hosts. Parasitic larvae - procercoids - develop in the body cavity (haemocoele) of copepods. Many fish species such as perch feed on cyclopoid copepods, including infected ones, and thus become the second intermediate hosts. The next larval phase - plerocercoid - develops in the internal organs (frequently the liver) and muscles of the second intermediate host. When the latter are consumed by pike (definitive host) the plerocercoids develop into adult worms in their intestine (Kuperman, 1973).

The life cycle of all the species of the genus *Triaenophorus* is quite similar. In the early independent phases of development (eggs and coracidia) no significant

differences are observed. Neither are there any significant morphological differences in the first parasitic phase of the life cycle - the procercoids - among the various species of *Triaenophorus*. The development of the procercoids of this genus takes place in the body cavity of copepods and no evidence of host specificity with reference to the first intermediate host has been found; the procercoids of the various species of *Triaenophorus* may develop in one and the same species of copepoda.

In the next larval phase of the life cycle however, the plerocercoid stage, which proceeds in the internal organs of the body or the muscles of different fish species [perch (*Perca fluviatilis*), smelt (*Osmerus eperlanus*), grayling (*Thymallus thymallus*), bulhead (*Gobio gobio*)], significant morphological difference are evident in the various species of the genus *Triaenophorus*. The attachment apparatus, which is the most important and typical taxonomic character of these cestodes, commences and completes development during the plerocercoid stage. The scolex and hooks of *Triaenophorus* species, which are also initiated and fully formed during this stage of development, retain their shape and size in the final or adult phase.

1.7. Aims and objectives of this study

This study has four central aims, namely,

(**i**). To make, for the first time, a detailed descriptive epidemiological analysis of the helminth and protozoan parasites of the population of the three-spined sticklebacks (*G. aculeatus*) in the River Wandle (a partially polluted tributary of the Thames).

(**ii**). To attempt to correlate patterns of infection with *S. solidus* in *G. aculeatus* with the available data on pollution levels in different sections of the River Wandle (The opportunity will also be taken to make a critical and detailed review of the literature on host - parasite systems as biological indicators of environmental pollution and biological indicators in general).

(**iii**). To carry out laboratory experiments to examine the impact of infection with *S. solidus* on the capacity of *G. aculeatus* to biotransform two phenols (phenol and

pentachlorophenol) and also examine the effect of parasitization on the toxic effects of the stressors towards the fish.

(**iv**). To investigate, using histological and biochemical methods in parallel, the impact of hepatic plerocercoids of *T. nodulosus* in perch (*Perca fluviatilis*) on the capacity of the host to biotransform phenol.

Chapter Two

2. General Materials and Methods

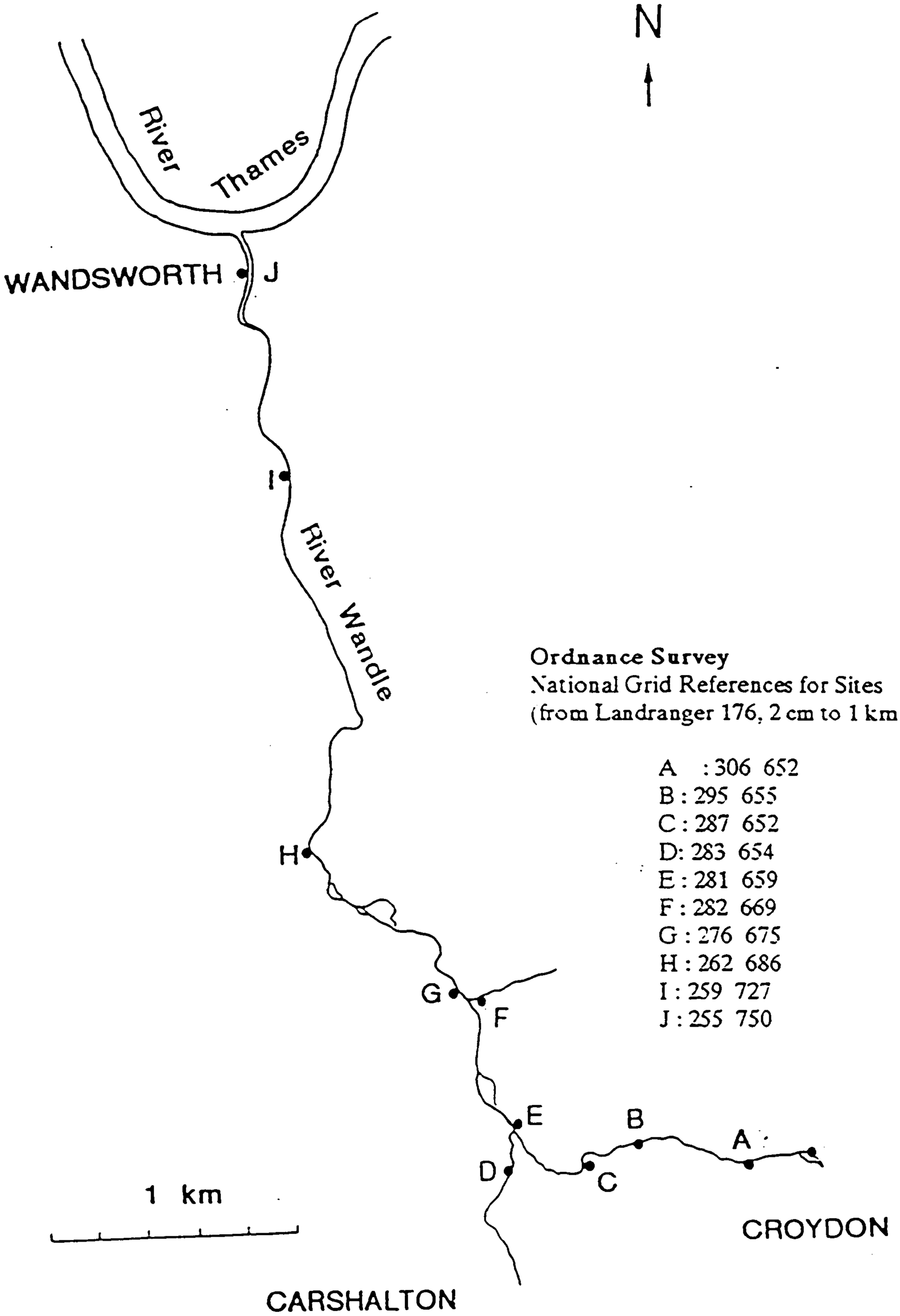
2.1. Introduction

The aim of this chapter is twofold. Firstly, it provides details of the rationale behind the sampling programme and secondly, it details the key laboratory methods employed throughout the investigative phases of this research. A major initial part of this research was to conduct a site assessment in order that suitable sampling sites could be identified for the purpose of this current study. The site assessment programme was principally undertaken on the River Wandle aided by the previous study of Chen (1992).

2.2. The River Wandle : A preliminary site assessment

Extending from Wandsworth to Croydon and beyond, the Wandle is a significant tributary of the River Thames (**Figure 2.1**).

In order to assess the availability of fish, an initial quantitative evaluation was carried out for several sites together with measurements of water temperature (**Table 2.1** and **Figure 2.2**). The stickleback populations in the River Wandle were ascertained to be predominantly a 'leiurus' population, mixed with a very small proportion of 'semiarmatus' morphs (Chen, 1992). This outline shows the abundance of the key stickleback species and those other "incidental" species that were encountered. This survey work was of considerable value to the present study when deciding which sites to sample and, more importantly, provided information on the presence and availability of the chosen fish species.



Ordnance Survey
National Grid References for Sites
(from Landranger 176, 2 cm to 1 km scale).

A : 306 652
B : 295 655
C : 287 652
D: 283 654
E : 281 659
F : 282 669
G : 276 675
H : 262 686
I : 259 727
J : 255 750

Figure 2.1: A map shows the 10 sampling sites in the River Wandle (Meng, 1992).

Table 2.1: Fish species obtained during the initial sampling assessment of River Wandle from Sept. to Oct.1992.

Site	Species		"Occasional" species sampled
	G. aculeatus	***P. pungitius***	
A	++	-	-
B	++++	-	-
C	+++	++	-
D	+++	+	*Cottus gobio*
E	+++	++++	*Cottus gobio, Noemacheilus barbatulus*
F	++	-	-
G	+	+	-
H	+++	+++	-
I	++	-	-
J	-	-	-

–	=	absent/not recorded
+	=	present (sporadic occurrence)
++ – +++	=	regular
++++	=	abundant

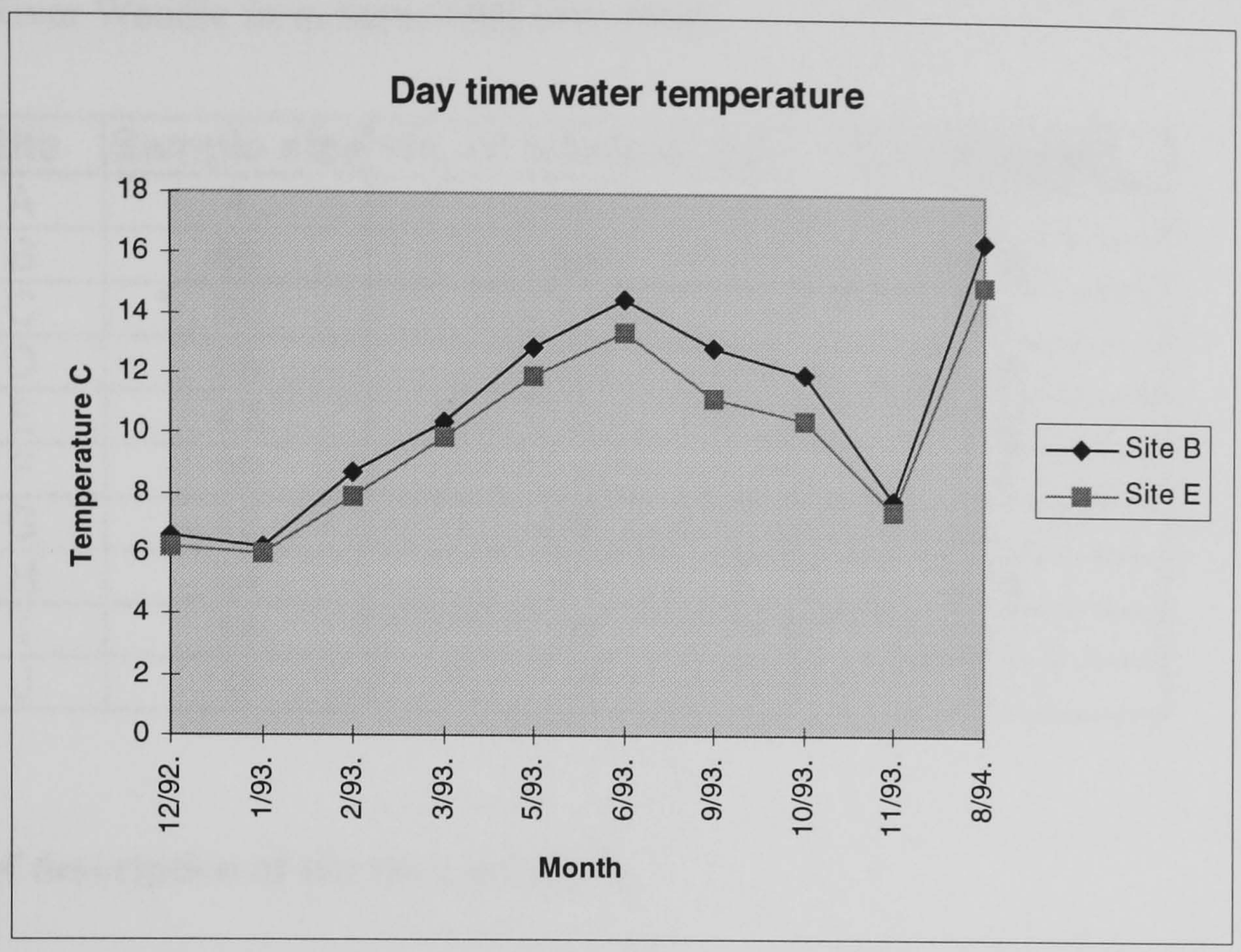


Figure 2.2: Day time water temperature, sampled on monthly basis from December 1992 to August 1994.

Since it was shown in a previous investigation (Chen, 1992) that in parts of the River Wandle the three - spined stickleback populations were heavily infested with the parasite *S. solidus* it was decided to continue to record the infection at each site. At sites A,B,C,D,E, F and H the stickleback was found to be predominant species and the incidence of infection by the *S. solidus* varied between 100% and 5.3% according to the site (**Table 2.2**). In sites G I and J the presence of the stickleback was irregular and sporadic and lacked infestation by the parasite of interest. The presence of the nine-spined species, *Pygosteus pungitius* was also observed at sites C,D,E, G and H. Other fish species noted at sites D and E include stone loach (*Noemacheilus barbatulus*) and bullhead (*Cottus gobio*). A brief description of each site examined during the preliminary site evaluation is given below.

Table 2.2: The prevalence of infection by *S. solidus* in the three-spine stickleback from the River Wandle from Sept. 1992 Oct. 1992.

Site	Sample size	No. of infected fish	% of infection
A	4	4	100
B	65	56	86.2
C	50	27	54
D	55	23	41.8
E	47	6	12.8
F	19	1	5.3
G	17	0	0
H	34	11	32.4
I	14	0	0
J	0	0	0

2.3. Brief description of the sites surveyed

Selection of suitable sites was considered to be important to the main framework of this study. The choice of site was heavily governed by

(i) availability and range of fish species,

(ii) accessibility of the sampling site and

(iii) availability of existing data on contaminant levels, disease and physical characteristics of the sites.

Though the study of Chen (1992) provided some guidance about each of these sites, the present work evaluated the viability of each site more thoroughly to determine which sites would best meet the needs of this study.

The sites named A through to J ranged from the confluence of the Wandle with the Thames to the upper reaches of the Wandle at Croydon and beyond. As **Table 2.1.** and **Table 2.2** demonstrate, the sites showed some differences in the fish species present, with sites A, I and J immediately being ruled out of this present investigation due to extremely low numbers of fish being available. By a process of elimination, sites B, C, D, E, F and H were possible sampling sources to provide the fish and parasites needed for the present work.

2.3.1. Site A Croydon Arm below Waddon Pond at Aldwich Road

This site is located downstream of a factory and there was a noticeable element of industrial activity in this area. Fish numbers were very low, therefore this site was regarded as not being suitable for the purposes of the current investigation.

2.3.2. Site B Croydon Arm, Beddington Park, Church Road

This is an urban site and had a large population of parasitised and non - parasitised three-spined sticklebacks and it provided an easy site to sample. The site was selected for future study.

2.3.3. Site C Croydon Arm, London Road/Manor Gardens

This site had similar characteristics to site B, but it was relatively inaccessible and the site was abandoned.

2.3.4. Site D Carshalton Arm, Mill Lane, Carshalton

Again, this site, was typically urban and preliminary assessment of the indigenous fauna revealed a large stickleback population. However, it is very difficult to get access to collect the samples thus rendering it unsuitable for the purposes of this particular investigation.

2.3.5. Site E Hackbridge

This is another urban site. Water movements were rapid. Sampling at this site was straightforward and access was not a problem. Fish availability and easy accessibility to this site made a good choice.

2.3.6. Site F Beddington sewage effluent/Goat Road

This site was considered to be poor sampling site with consistently low number of sticklebacks being obtained. Due to sewage activities it was decided that it would be unusable for the purpose of regular, seasonal sampling.

2.3.7. Site G Below the junction of Beddington sewage effluent

This site was also considered to be a poor sampling site with low number of sticklebacks being obtained. Therefore it was decided not to use this site for the present study.

2.3.8. Site H Morden Cottage, Morden Hall Park

This site is owned by the National Trust. Obtaining a permission to collect fish samples needed a long procedure. This site was therefore excluded from the sampling program.

2.3.9. Site I Steerforth Street, Summertown, London

Initial visual observation of this site identified numerous hazards, including, discarded sections of vehicles, trolleys and a vast amount of other waste and debris. Accessing the site was not easy and sampling was extremely difficult. Additionally, the water quality was markedly poorer and few sticklebacks were recovered. A lack of fish and very hazardous sampling automatically disqualified this site from being used further.

2.3.10. Site J River Wandle/River Thames confluence, Wandsworth

This site is generally an area of increased depth, devoid of any sticklebacks and of diminished water quality. Sampling here was extremely difficult, and dangerous. However, the lack of the required test species meant that this site was of little use for this study and it was therefore abandoned.

It was found that the River Wandle possesses no accessible parasite-negative control sites and after thorough examination, only two sites were chosen for the continuation of the present work. Site B (**Figure 2.3**) located at Church Road, Beddington Park, Croydon (**GR 295 655**) presented a highly infected stickleback population while site E (**Figure 2.4**) located at Hackbridge, Croydon (**GR 281 659**) provides a much lower prevalence of parasite infection.

Figure 2.3: Junction of two arms at Hackbridge, Croydon (site E) GR.281-659.

Figure 2.4: Church Road, at Beddington Park, Croydon (site B) GR. 295-655.

2.4. Test Species

2.4.1. Fish and parasite collection

Three-spined sticklebacks (*G. aculeatus*) were collected from sites B and E using a standard procedure involving dip-netting. They were quickly transferred to 500ml, darkened polystyrene pots and placed in a Carver® cool-box. Prior to placing fish in each pot the water was "saturated" using technical grade oxygen (BOC gases, Wembley, Middx.). The fish were then transported to the laboratory aquarium facility where they were transferred to a stock holding system for later use in chemical experimental studies. Those selected for investigation of parasite infection were sacrificed and preserved in 10% formalin and examined for ecto and endo parasites.

Perch (*Perca fluviatilis*) were collected from the NRA Fisheries Laboratory, Denton, near Reading, Berkshire, England. Perch were obtained from a combination of seine and dip-netting techniques by the NRA Fisheries staff and then transported to the laboratory holding facility.

The distribution and occurrence of parasites in the stickleback were monitored using fish that had been sacrificed by a blow to the head and preserved in 10% formalin solution. Parasites were collected from the peritoneal cavity and other sites of the body transferred into and preserved in 10% formalin solution and were maintained in glass specimen jars until required for examination.

2.4.2. Fish Maintenance

Both sticklebacks and perch were held in a semi-static aquarium tanks that contained 8L of water. Each tank was lined with acid-washed sand, and tanks were gently aerated using mains compressed air, delivered by an all-glass pipette delivery system. Air was filtered using a filtration apparatus to remove any contaminants, principally copper, from the mains air supply pipes.

Fish were maintained in the aquarium system which was housed in a constant temperature facility where the temperature was kept at 4°C. This room operated on a photoperiodic regime consisting of 12Light : 12Dark photoperiod.

Fish were allowed to acclimatise to the conditions for a period of not less than one week. Tanks were also sealed using a tightly-fitting glass lid with only a hole for the insertion of the pipette air-line. Fish were regularly inspected and physical symptoms of ill-health and mortality noted. During the period of holding mortality among the fish never exceeded 1% of the population (**Figure 2.5**).

Three - spined sticklebacks length and weight were recorded. The length (cm) being from the most anterior end of the snout to the most posterior end of the caudal fin. The total weight (g) of the fish includes that of parasites. Separate weights of the body mass of fish and the total mass of parasites per fish were also recorded.
In a number of situations, mean sizes of fish in different samples and plerocercoids were compared statistically using student's t - test.

2.5. Analytical Methods

2.5.1. Staining methods for parasites and fish tissue sections

All samples of parasites and fish tissues for examination under the microscope were firstly fixed in 10% formalin. Borax-carmine solution was used to stain *Gyrodactylus*, *Trichodina* and *Proteocephalus* specimens by removing the parasites from the formalin and washing them several times in water. They were then placed into 70% alcohol for about 1hr, after which they were placed in Borax - carmine stain for 5 - 10 minutes, the staining time varied from one species to another. Following this they were transferred to 50% acid alcohol, to aid the differentiation of individual organs. After that the parasites were transferred to 70% alcohol for 10 - 15 minutes. The final stages involved a series of washings in alcohol with 90% alcohol for 10 - 15 minutes, two changes of 100% alcohol, for 10 - 15 minutes each. Then xylene for 15 minutes to obtain clear specimens and then half a mixture of xylene and Ralmount over night. The specimens were now ready to mount on a slide using Ralmount and then left to dry. Alcian Blue stain was applied to liver sections to stain the

mucopolysaccharides. Mallory - Trichrome stain was used to stain the liver musculature and collagen. Haematoxylin and Eosin stain was used to stain the white cells infiltration (inflammation) (see Chapter 5 for details).

2.5.2. High performance liquid chromatography (hplc) determination of phenol and pentachlorophenol (PCP)

High performance liquid chromatography (hplc) has been employed for the separation of phenol and PCP and their biotransformed products in the bile of sticklebacks and also the glucuronide synthesised from phenol by liver homogenates of perch these methods are detailed in chapters 4 and 5.

The hplc used in the present study was a Varian 5000 liquid chromatograph (Varian Associates Inc., Walnut Creek, Ca 94598, U.S.A.) which contained a variable UV-detector that was operated at 254nm, a C-18 reversed phase column (Techsil 5C18 4.6 x 250mm, Greyhound, W. Birkenhead, Merseside, U.K.) and an isocratic solvent delivery system. The chromatograph was connected to a Hewlett Packard integrator, type 3390A (Anachem, Luton, Beds., U.K.).

The eluents used in this present study were acetonitrile and methanol which were combined with water in the proportion of 1 : 1 : 2, v/v (Layiwola, 1988). These are the most popular organic solvent for hplc as they have a relatively low ultraviolet cut-off (190nm and 210nm respectively) and are readily available with sufficient purity.

The operating conditions were as follows:

Pressure - Max :	350 atmos.
Min :	10 atmos.
Flow rate :	1.2 ml/min.
Wavelength :	254nm
Recorder :	0.05 Aμ/mV.
Time constant :	0.5 sec.
Chart speed :	1.0 cm/min.

2.5.3. Quantit ative measurements by hplc. Internal standard method

Samples were quantified on the basis that the amount of substance present is proportional to its peak area/height. Calibration was performed, by correlating the peak area/height with a known sample concentration.

An internal standard has to be a component that can be completely resolved, but is not present in the sample mixture. In this work 2,3,5,6 - tetrachlorophenol was used as the internal standard for phenol and its biotransformed product and phenol was used for pentachlorophenol and its metabolite. The method entails the addition of a known constant amount of the internal standard to varying concentrations of the phenols under test. Then the ratios of peak height of the phenols to that of the internal standards were determined and these were subsequantly used as a means of quantifying phenols in fish samples (Willard *et al*, 1981).

Thereafter the same amount of internal standard is added to the test sample before injection on to the hplc column. The peak height ratio of the peak of interest is measured and the concentration is then determined from:-

$$C_{analyte\ (mg/l)} = \frac{H_{analyte\ (cm)} * C_{(I.S)}}{H_{(I.S)}}$$

Where C = concentration and H = peak height

I.S = internal standard

2.6. Experimental Methods

2.6.1. Toxicity testing procedures

Several types of toxicity testing procedures have been developed, and these include acute, sub - acute, and chronic studies (Standard Methods, 1980; OECD Document 203, 1981). The major difference between these tests is in the nature of the

concentration employed and the length of time of exposure to a chemical. All tests share common characteristics as each requires groups of healthy animals, acclimatised under suitable conditions and exposed to different concentrations of the test chemicals, including a control group not exposed to the chemical. After the treatment, the animals are then carefully observed for any symptoms of toxicity. Acute toxicity has been defined by Hagan (1959) and Sprague (1973) as the adverse effects occurring in an organism within a short term of administration of dose(s) over a period of 24h to 7days. The most frequently used acute toxicity test involves determination of the median lethal dose/concentration of the chemical. This has been defined as a "statistically derived expression of a single dose (or exposure concentration) of a material that can be expected to kill 50% of the test population" (Gehring *et al*, 1973). It is referred to as LD_{50}/LC_{50} with the time period stipulated.

In this study the short - term toxicity tests were employed which involved 96h exposures of stickleback to phenol and pentachlorophenol. The procedure adapted is based on previous work by Layiwola (1988) and Furay (1994). The toxicity testing system consisted of five exposure concentrations and one control. All the experimental tanks were made of glass (30L Gallenkamp Scientific tanks). Each tank contained 10L of dechlorinated tap water to which the fish had been previously acclimated . The fish were kept in well aerated conditions and to minimise evaporation of the test compounds the tanks were sealed with a tightly-fitting glass lid. The exposure system was housed in a constant temperature room at a temperature of 4°C and under a light regime of 12h light and 12h dark (see **Figure 2.6**).

Stock solutions (1000µg/ml) of phenol and PCP were prepared in distilled water, in the case of PCP it was first dissolved in a minimal volume of 1M NaOH, and then added to distilled water in a volumetric flask adjusted to pH 8.5 using 1M HCl and made up to volume. The appropriate amount of stock was added to each tank and 8 (or in some experiments 16) fish were introduced. Previous work had shown that in order to maintain exposure concentration above 80% of the nominal values the immersion medium needed to renewed after 48hr. To minimise unduly stressing the fish this was done by removing and renewing 80% of the test solution. Fish mortality, and any adverse effects were monitored daily and the cumulative percentage mortality

was recorded after each 24hr period. Dead fish were removed to prevent fouling of the test medium. The control groups were treated in exactly the same way except that no test compound was added. Fish were not fed with food before and during the exposure periods. In the toxicity experiments reported in this work there were no mortalities in all control treatments.

2.6.2. Identification of phase II phenyl glucuronide conjugates in the bile of stickleback

Enzymatic hydrolysis was used to identify the presence of the glucuronide conjugates of phenols in the bile of fish. β- glucuronidase (1.0ml equivalent to 5000 units per ml of a bovine liver preparation buffered at pH 5, type 'glucurase') (Sigma Chemicals Co. Ltd., Poole, Dorset, U.K) was added to duplicate samples of bile (3.0ml, pH 5) to which D-saccharic acid - 1,4 lactone (20mM, 3.0ml) was added to the control only. Controls and samples of bile from exposed fish were then incubated at 37°C for 16h in a water bath with agitation of the incubation medium. Following this period of incubation with the hydrolytic enzyme the incubate was diluted with distilled water and then centrifuged in a MSE Centar 2 (Fisons scientific apparatus, Loughborough, Leics., U.K) at 4500 rpm for 10 min to separate precipitant and the unwanted proteins. The supernatant was then retained and was analysed using the previously described isocratic hplc chromatography method. Disappearance of the peak, after β - glucuronidase hydrolysis confirmed the presence of the glucuronide conjugate in the bile of the fish. This technique was used in all cases to identify glucuronide conjugates.

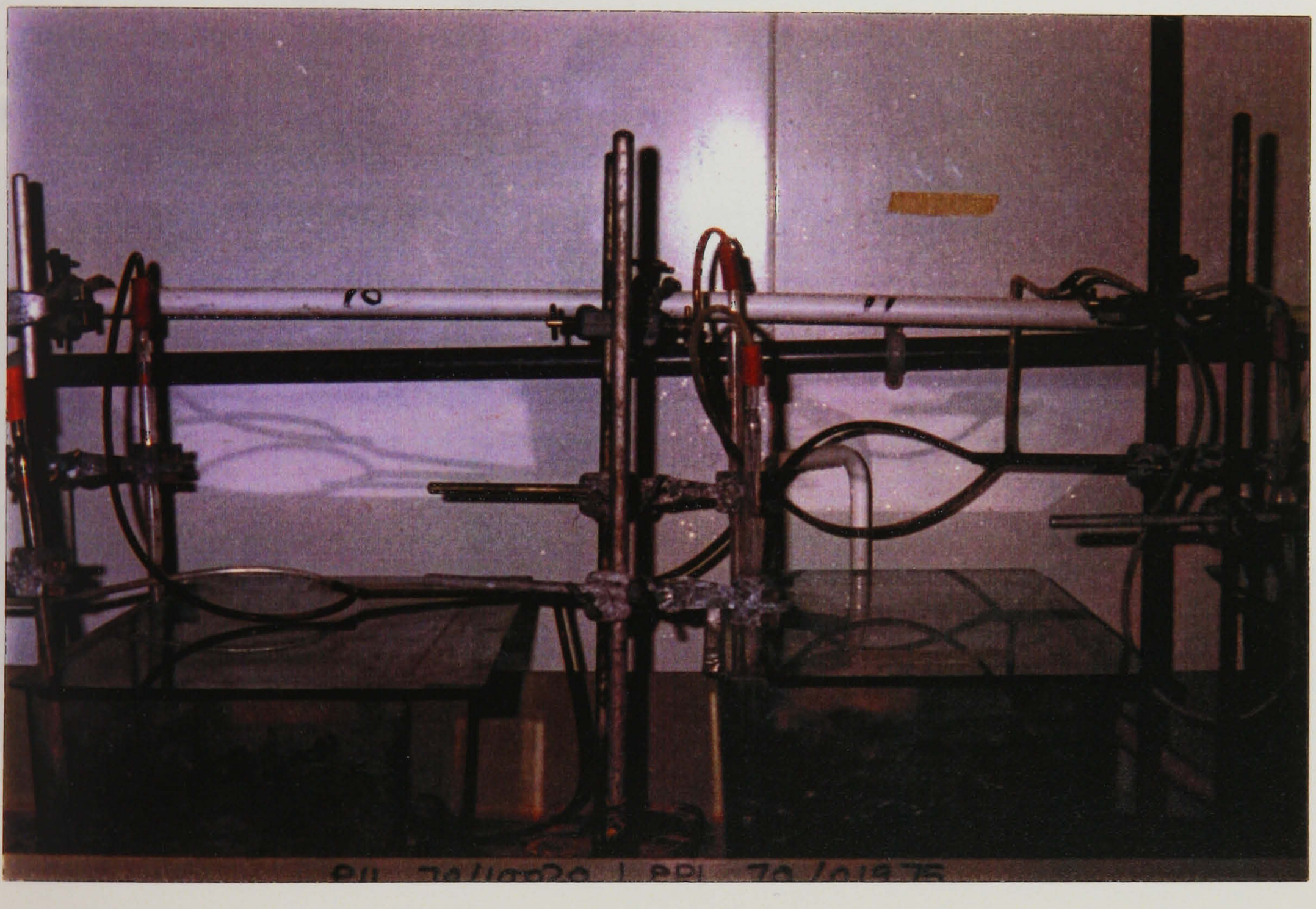


Figure 2.5: A photograph showing the stock accommodation system.

Figure 2.6: A photograph showing the toxicant exposure system.

Chapter Three

Studies on the parasites of Wandle sticklebacks

3.1. Introduction

This chapter provides a descriptive epidemiological analysis of parasitic infections of sticklebacks (*G. aculeatus*) from the two sampling areas in the River Wandle which were defined and described in **Chapter 2**. In parasite biotic terms one site, site B, was characterised in September 1992 by relatively high levels of infection with *Schistocephalus solidus* while site E was characterised by relatively low levels. By means of standardised fish sampling at both sites on 10 occasions between December 1992 and August 1994 a picture has been built up of :-

1) The changing population density and population structure of the sticklebacks over that period at both sites.

2) The changing infection load of sticklebacks at both sites with respect to the two cestodes *S. solidus* (larval plerocercoid infection) and *Proteocephalus filicollis*, the monogenean *Gyrodactylus arcuatus* and the two protozoan parasites *Trichodina megamicronucleata* and *Glugea anomala*. Most attention has been paid to infection with *S. solidus* plerocercoids and for this parasite it has been possible to provide additional information on the size structure of plerocercoid populations.

As indicated earlier, there is little information available on parasitic infection in Wandle sticklebacks, in spite of the fact that the Wandle is a significant tributary of the Thames. An earlier preliminary report, that of Chen, (1992), produced some ecological and epidemiological data on variations in parasitic infections in Wandle sticklebacks. However, most of this previous work was confined to the pseudophyllidean cestode, *S. solidus*.

In the sections of this chapter which follow, first, the changing population structure of sticklebacks will be described and then each of the five parasites that have been studied will be dealt with in turn. In each case, after an introductory section dealing

with the background literature on that parasite, results will be presented where appropriate, on changing patterns of parasite prevalence, intensity and mean intensity in relation to differences between the two sampling sites, seasonal differences and host size - specific differences. In some cases it has been possible to analyse the possible impact of parasite load on the hosts by measuring the parasitic index and condition factor (see Materials and Methods, section 3.2.3 for definitions).

3.2. Materials and Methods

3.2.1. Standardised fish sampling method

Two sites were sampled on the River Wandle at 10 collection dates between December 1992 and August 1994. Sampling was conducted using a standardised regime of fish collection, using a standard sweep technique involving collection by dip net. Each site was sampled on each occasion for a period of 1.5hr so that sampling effort was standardised. Fish were collected and then transferred to aerated collection containers.

3.2.2. Standardised fish analysis for parasite retrieval

Fish which were preserved in 10% formalin solution were examined for any ectoparasites and the presence of cysts using a Kyowa (Tokyo) model SDZ dissection microscope. Basic measurements of length and weight were recorded from the fish collected at both sites investigated. The total length (cm), from the most anterior end of the snout to the most posterior end of the caudal fin was recorded for each fish. After surface blotting of the fish, several weights were recorded for each fish. The total weight (g) of the fish, that is weight of fish and parasites was recorded. The weight of the body mass alone was also recorded as was the total mass of parasites alone per each fish. This gave rise to three weight categories being described, these being, weight of fish + parasites, weight of fish only and weight of parasites only (these differentiations are only of significance with respect to *S. solidus* infections).

3.2.3. Derivation of indices used for interpretation of the parasite data

Numerical analysis was performed on the raw parasite data to provide secondary indices. These were parasitic index, intensity, mean intensity, prevalence and fish condition factor (Arme and Owen, 1967). The derivation of these expressions is described in the following.

a): Parasitic index

= Weight of parasite (g) /Weight of parasite (g) + Weight of host (g)* 100

b): Intensity

= Mean number of parasites per infected fish

c): Mean intensity

= Mean number of parasites per fish (including uninfected fish)

d): prevalence

= No. of infected fish / Total no. of fish per sample * 100

e): Condition factor

= Weight of fish (g)/ Length (cm) ^{3}* 100

3.3. Results

3.3.1. Population structure of sticklebacks at sites B and E

The data from each of the seasonal samples is considered in terms of the total number of fish per sample and the length frequency classes of fish at each sampling point. This data is conveniently summarised in **Figures 3.1 - 3.11** for site B and **Figures 3.12 - 3.22** for site E.

Figures 3.1 (site B) **and 3.12** (site E) show that sticklebacks were found using the standardised sampling procedure at both sites and at all collection dates. Absolute sample sizes varied between 10 and 59. Although there were slight differences between the two sites there was a general pattern in terms of fish numbers, which

were highest between August and November, lowest in May and June and variable and intermediate in level between December and March.

When size class frequencies of fish are examined (see **Figures 3.2 - 3.11** for site B and **Figures 3.13 - 3.22** for site E) (see **Tables A1** and **A2** in **Appendix 3** for raw data) it is clear that there are differences in the size structure of the fish from the two sites. On average, fish at site B are slightly larger than those at site E (mean length at site B = 3.8cm, mean length at site E = 3.2cm). Underlying this difference in average size is a clear difference in the changing proportions of fish in the different size classes through time. This difference is exposed in **Table 3.1** which shows the changing percentage of fish in the lower two size categories (1 - 1.9cm and 2 - 2.9cm) at both sites. At site B there are no fish in these categories between January and May. Thereafter the percentage jumps to 82% in June before gradually declining to 7% in December. It seems likely that this increase corresponded with the recruitment of young - of - the - year into the population.

A comparison of mean numbers of fish recovered in the different size categories form sites B and E throughout the period of study (Table 3.1a and 3.1b). With the exception of size categories 4 - 4.9cm and 6 - 6.9cm in which significant differnces in the mean numbers of fish recovered from sites B and E ($p = 0.049$ and $p = 0.040$ respectively) were found, there were no significant differences in the mean numbers of fish at sites B and E in the other size categories.

Table 3.1a: Means and standard errors (SE) of fish numbers in different size classes at sites B and E.

Size class (cm)	Mean Site B	Mean Site E	SE Site B	SE Site E
1 - 1.9	2.5	2.6	1.5	1.1
2 - 2.9	9.1	15.0	3.7	2.6
3 - 3.9	13.2	8.9	2.8	1.8
4 - 4.9	10.1	5.0	2.2	0.75
5 - 5.9	3.1	1.4	0.85	0.45
6 - 6.9	1.5	0.4	0.43	0.22
7 - 7.9	0	0	0	0

Table 3.1b: T - test and confidence interval between each size classes of the sticklebacks at sites B and E.

Size class (cm)	T - test site B - site E	Probability site B - site E	Degree of freedom site B - site E
1 - 1.9	- 0.05	0.96	11
2 - 2.9	- 1.32	0.21	16
3 - 3.9	1.27	0.22	15
4 - 4.9	2.22	0.049	11
5 - 5.9	1.77	0.10	13
6 - 6.9	2.28	0.04	13
7 - 7.9	0	0	0

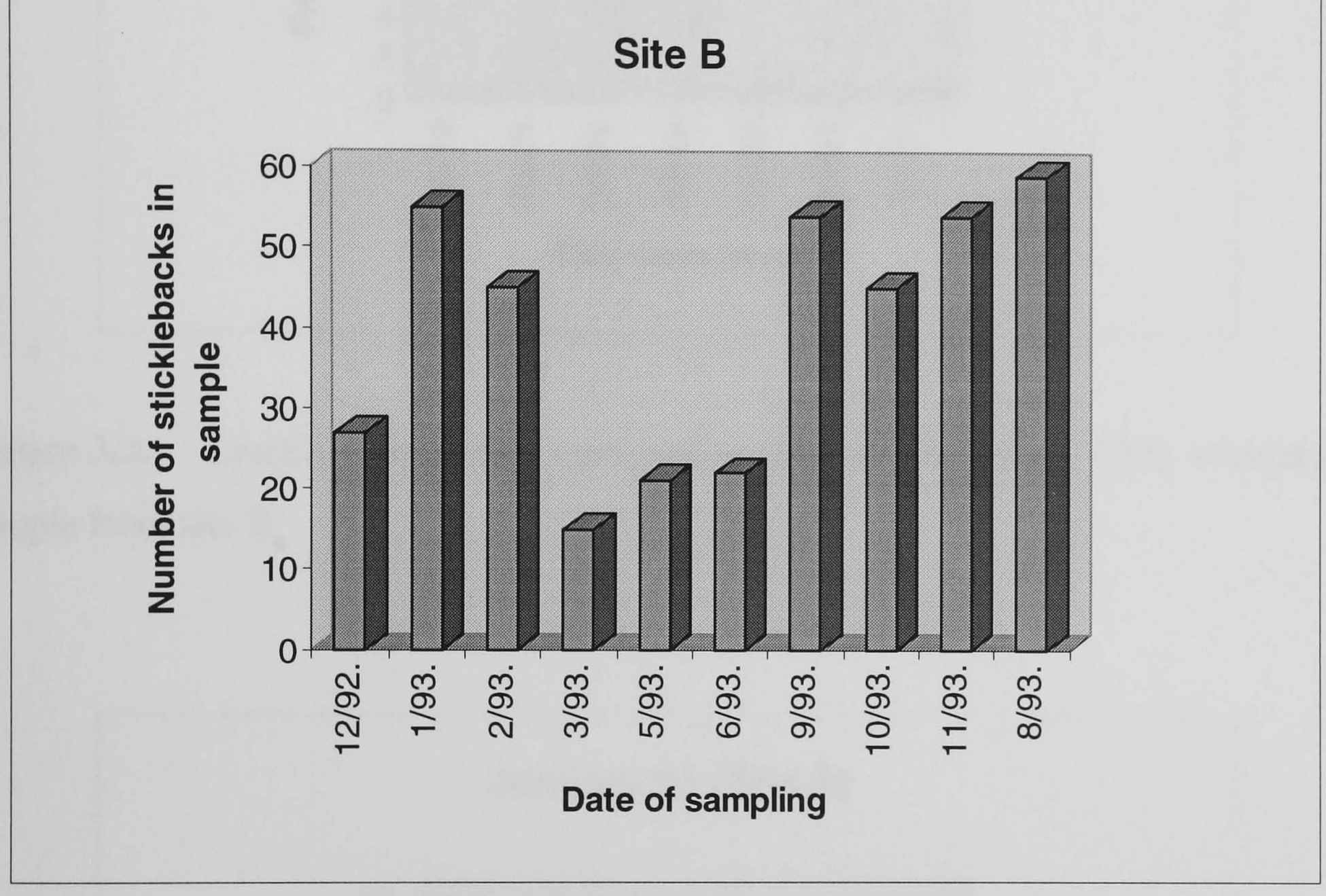


Figure 3.1: Numbers of sticklebacks per seasonal sample at site B.

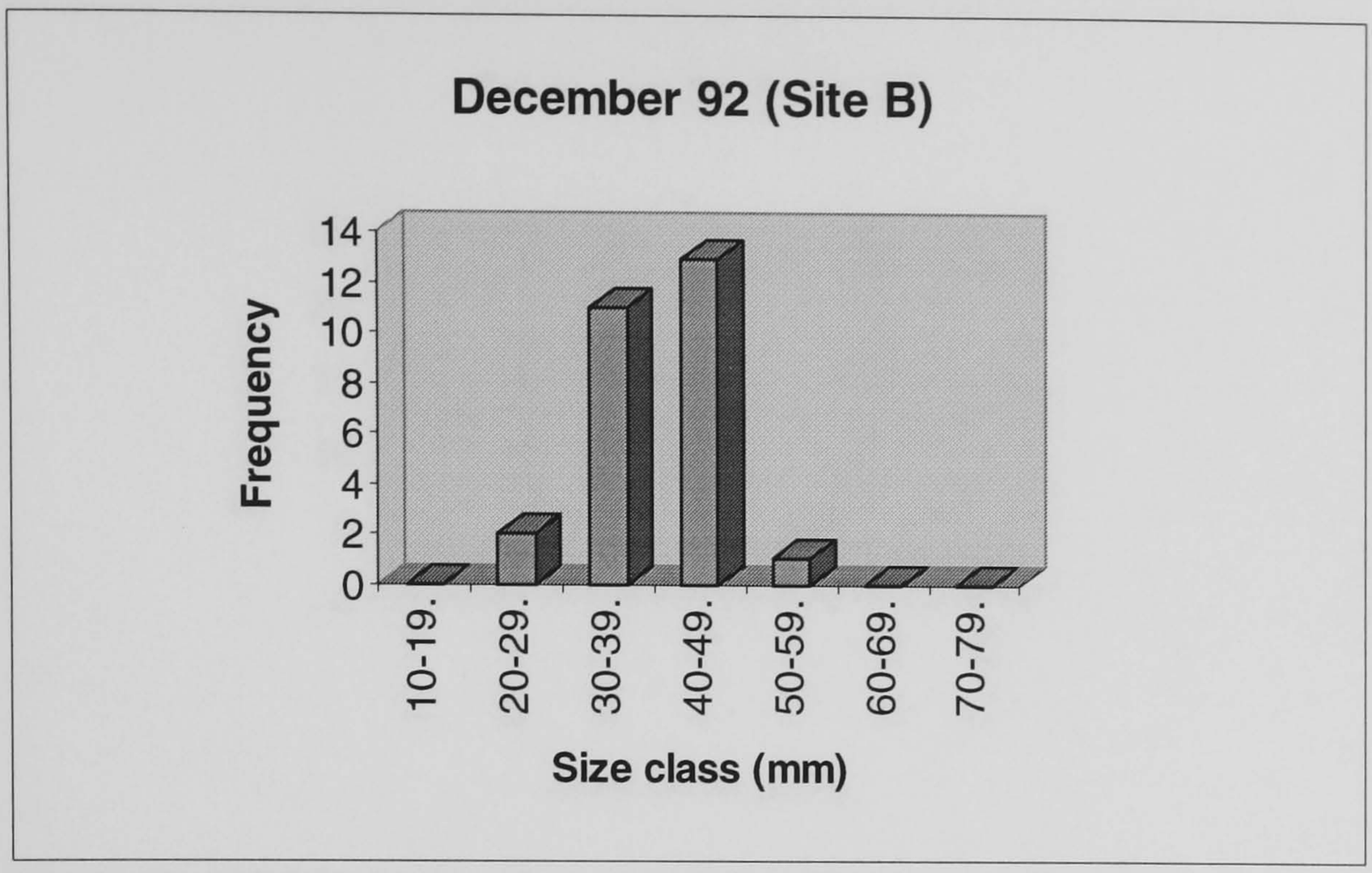


Figure 3.2: Length - frequency composition for the December 1992 stickleback sample from site B.

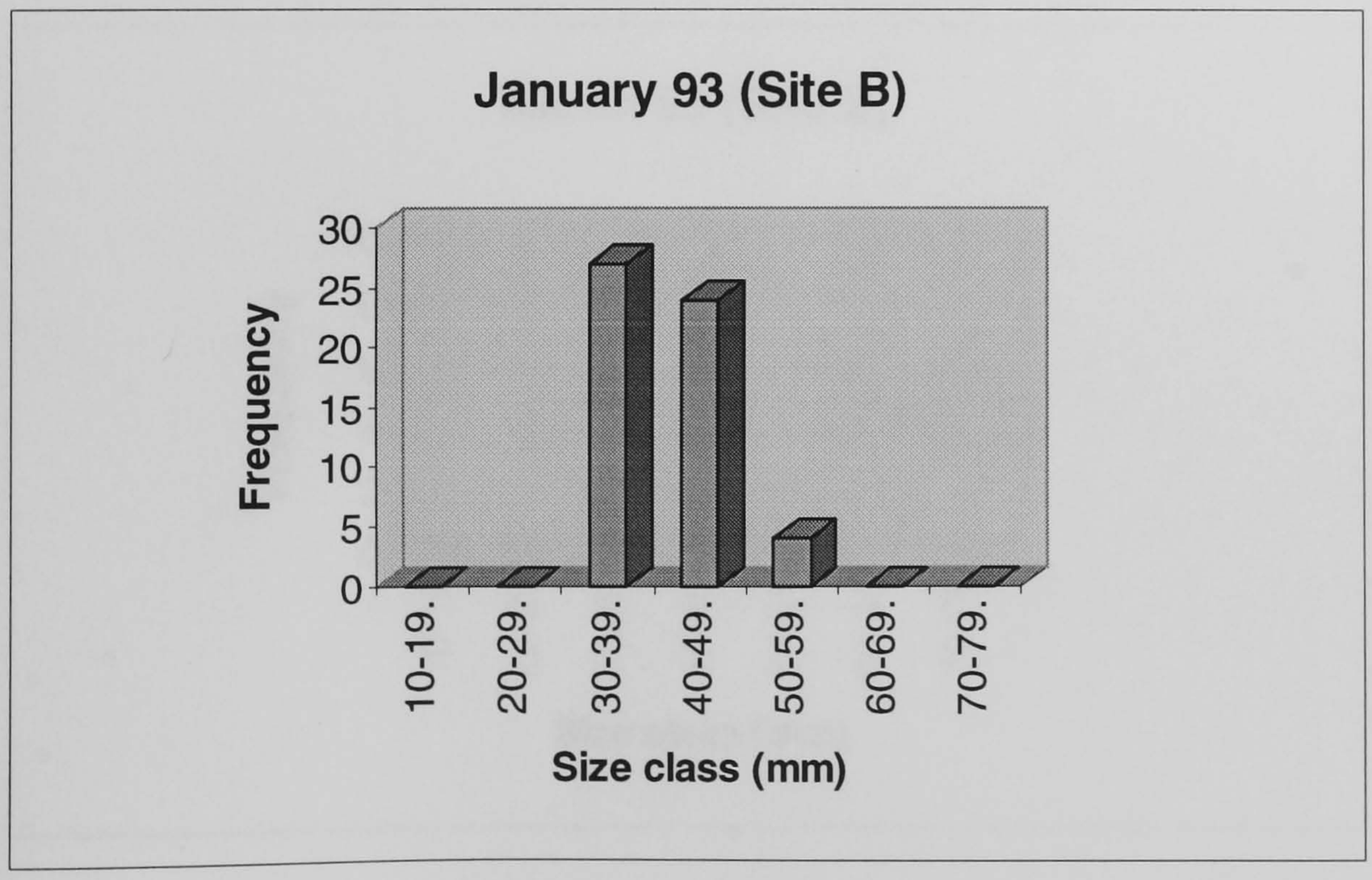


Figure 3.3: Length - frequency composition for the January 1993 stickleback sample from site B.

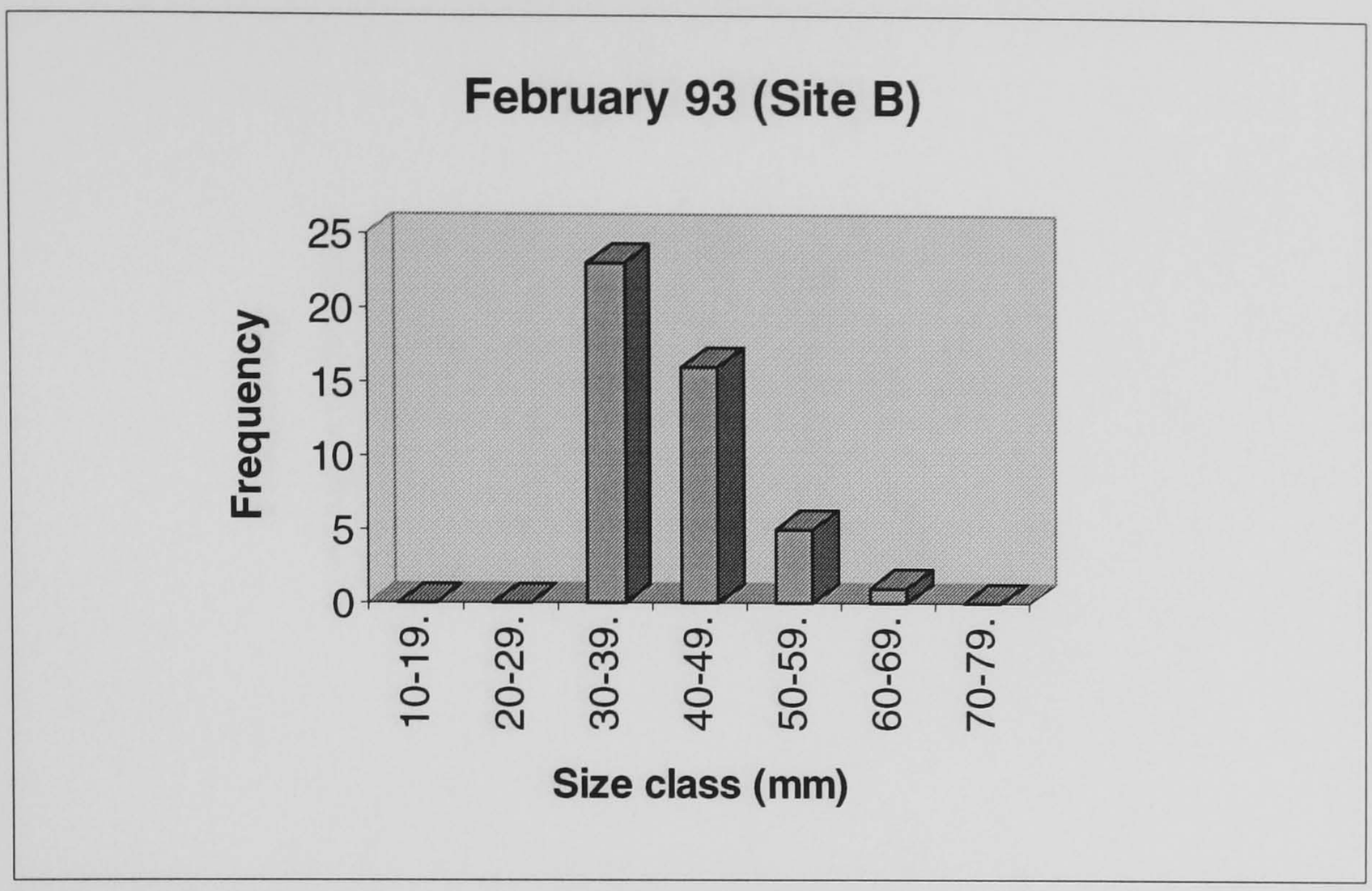


Figure 3.4: Length - frequency composition for the February 1993 stickleback sample from site B.

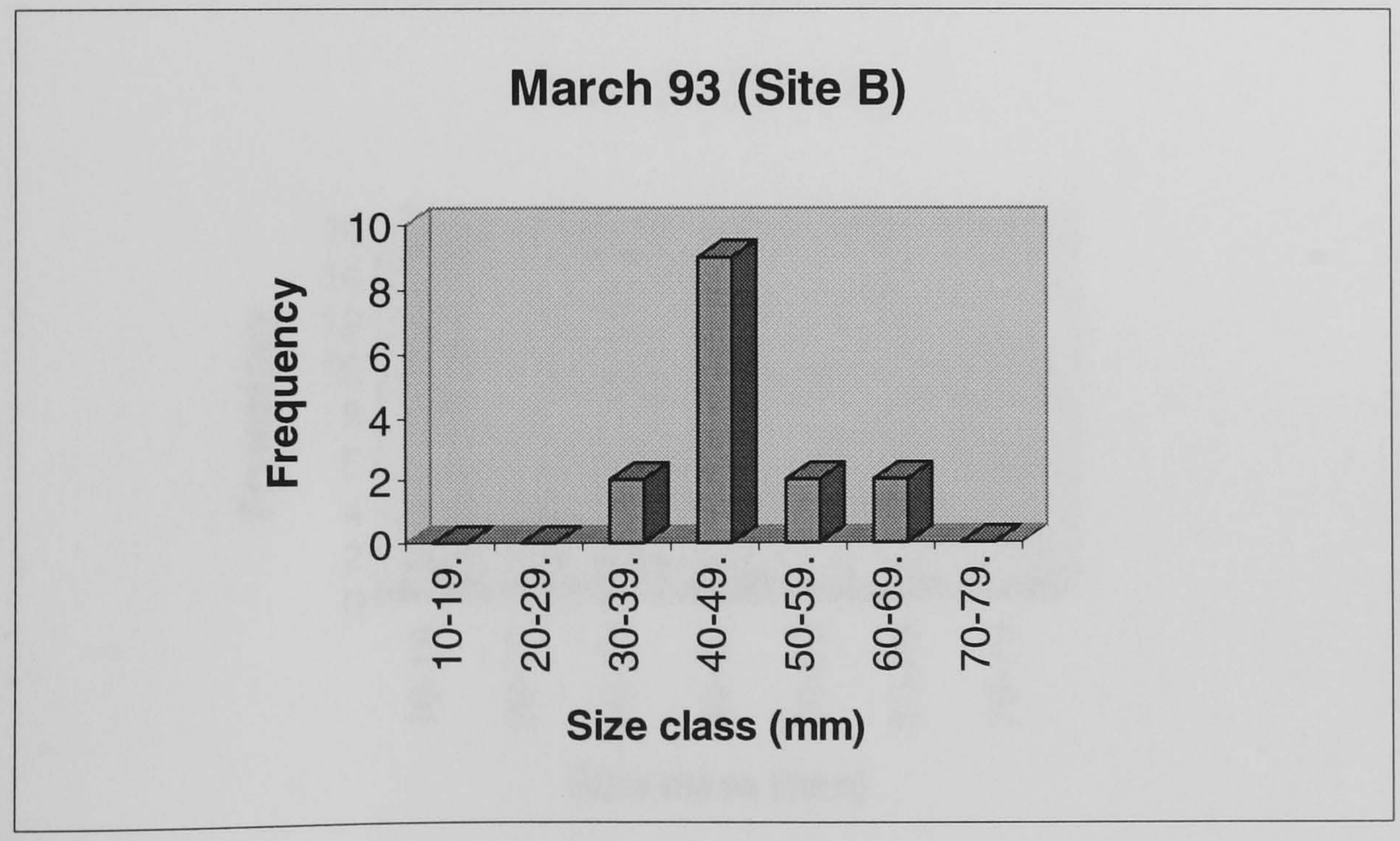


Figure 3.5: Length - frequency composition for the March 1993 stickleback sample from site B.

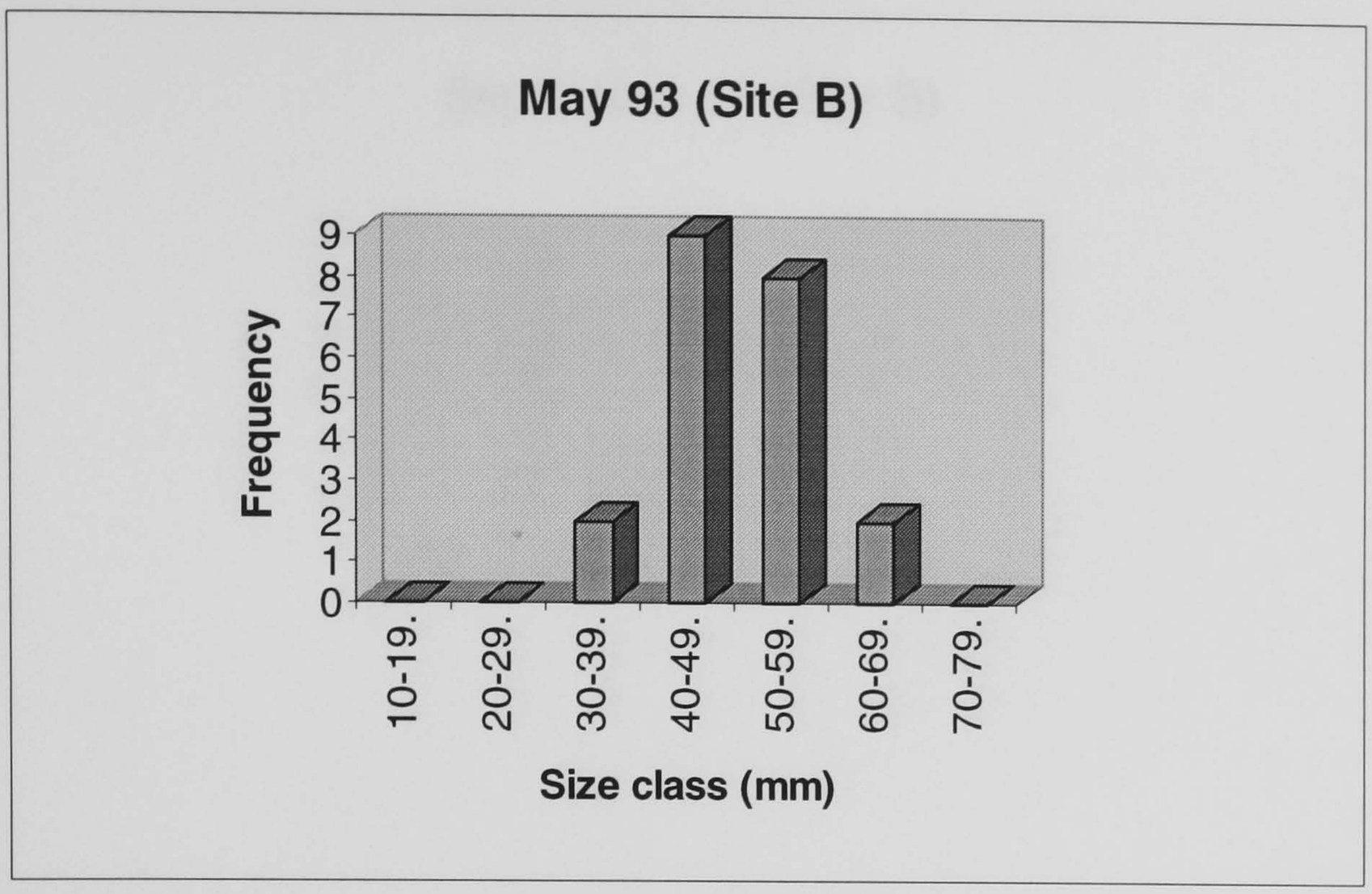


Figure 3.6: Length - frequency composition for the May 1993 stickleback sample from site B.

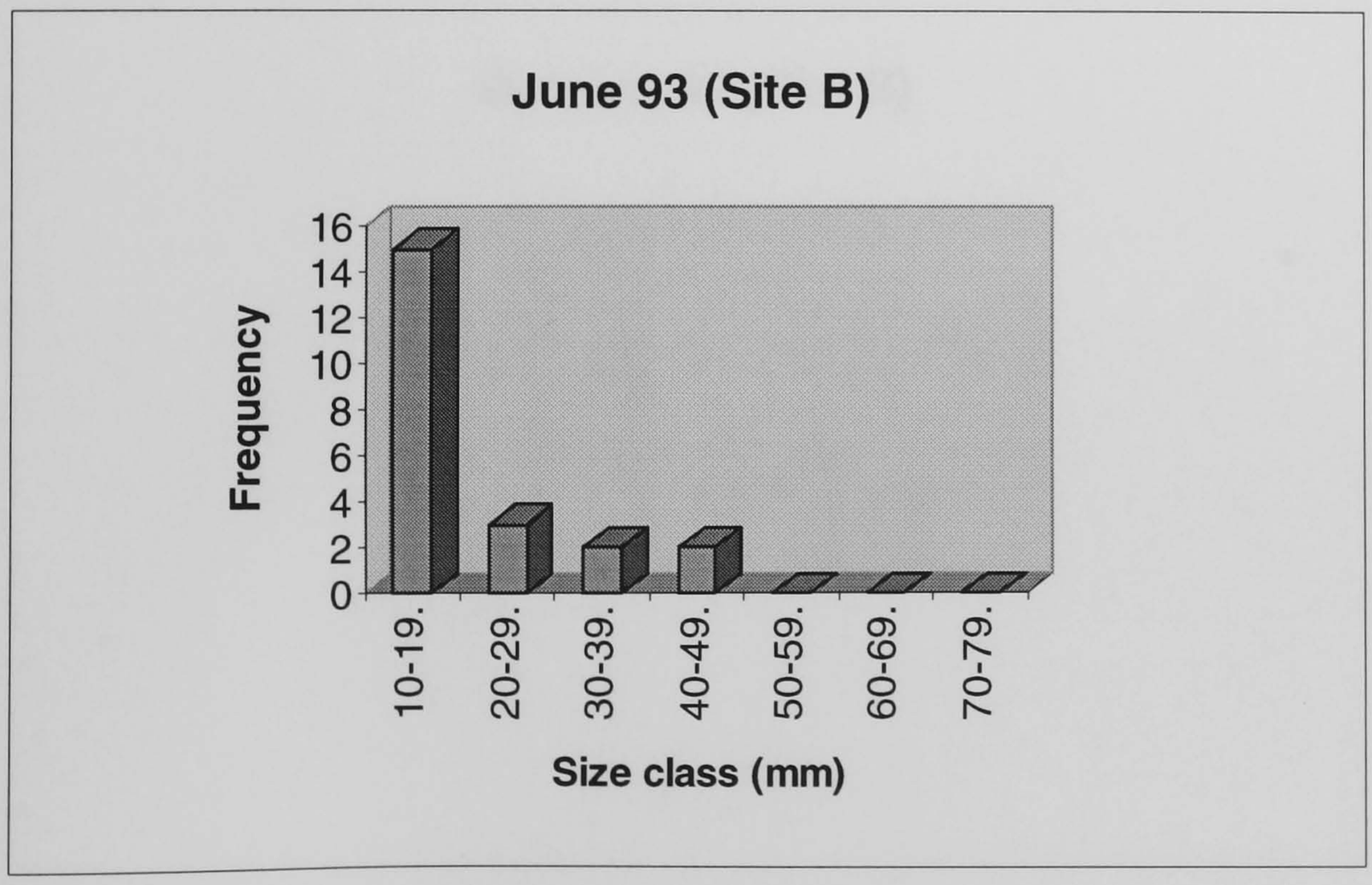


Figure 3.7: Length - frequency composition for the June 1993 stickleback sample from site B.

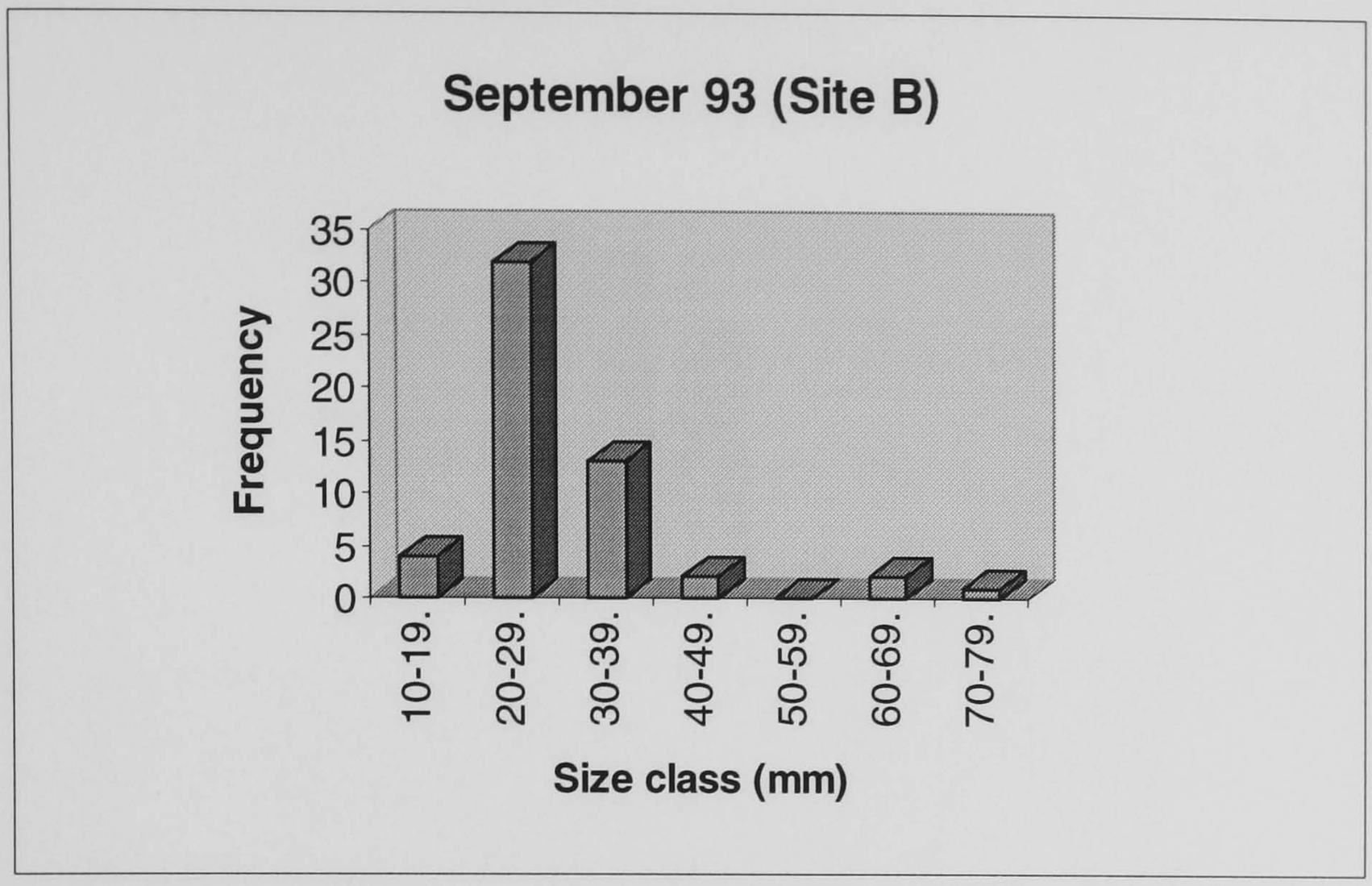


Figure 3.8: Length - frequency composition for the September 1993 stickleback sample from site B.

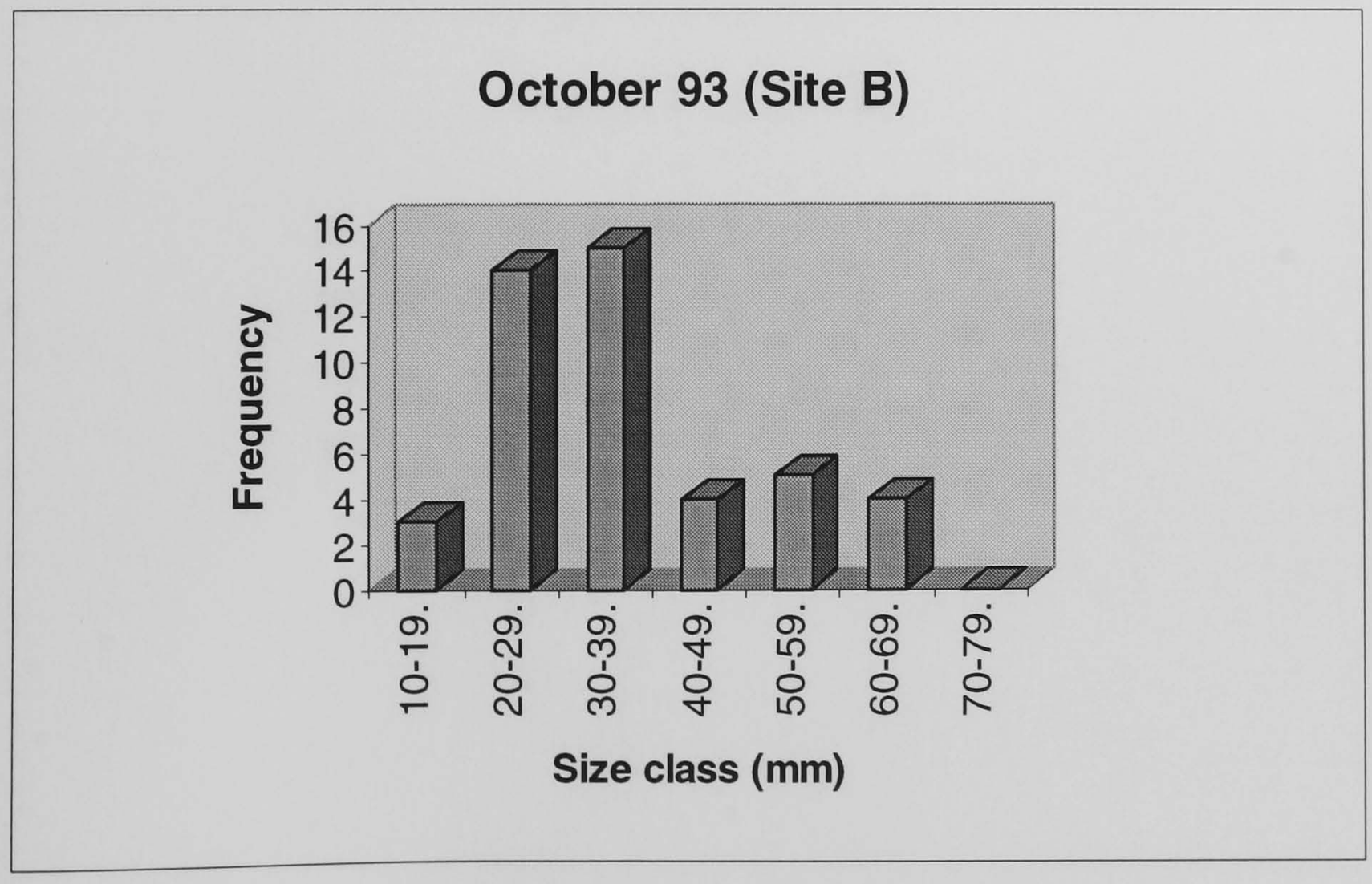


Figure 3.9: Length - frequency composition for the October 1993 stickleback sample from site B.

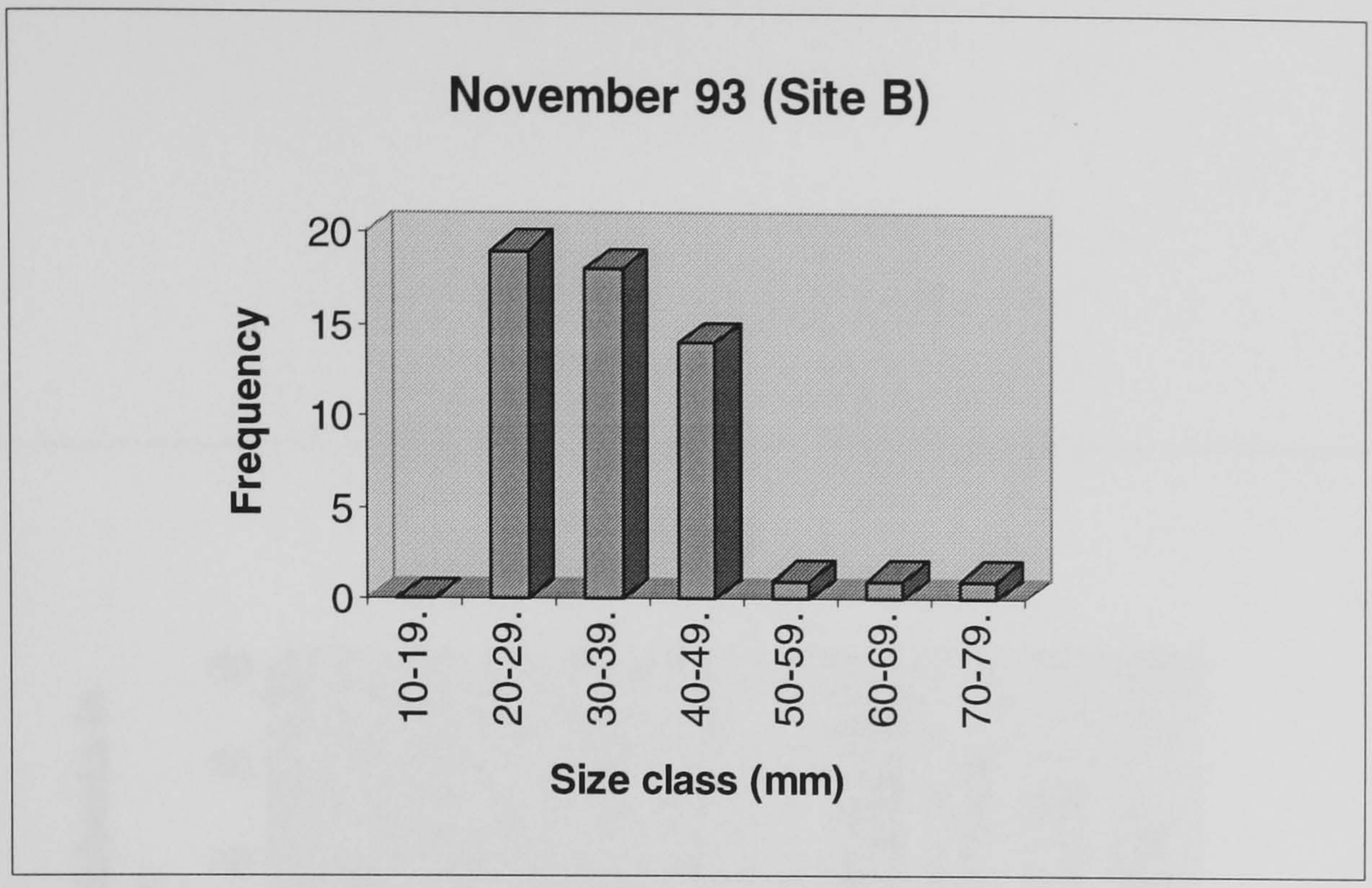


Figure 3.10: Length - frequency composition for the November 1993 stickleback sample from site B.

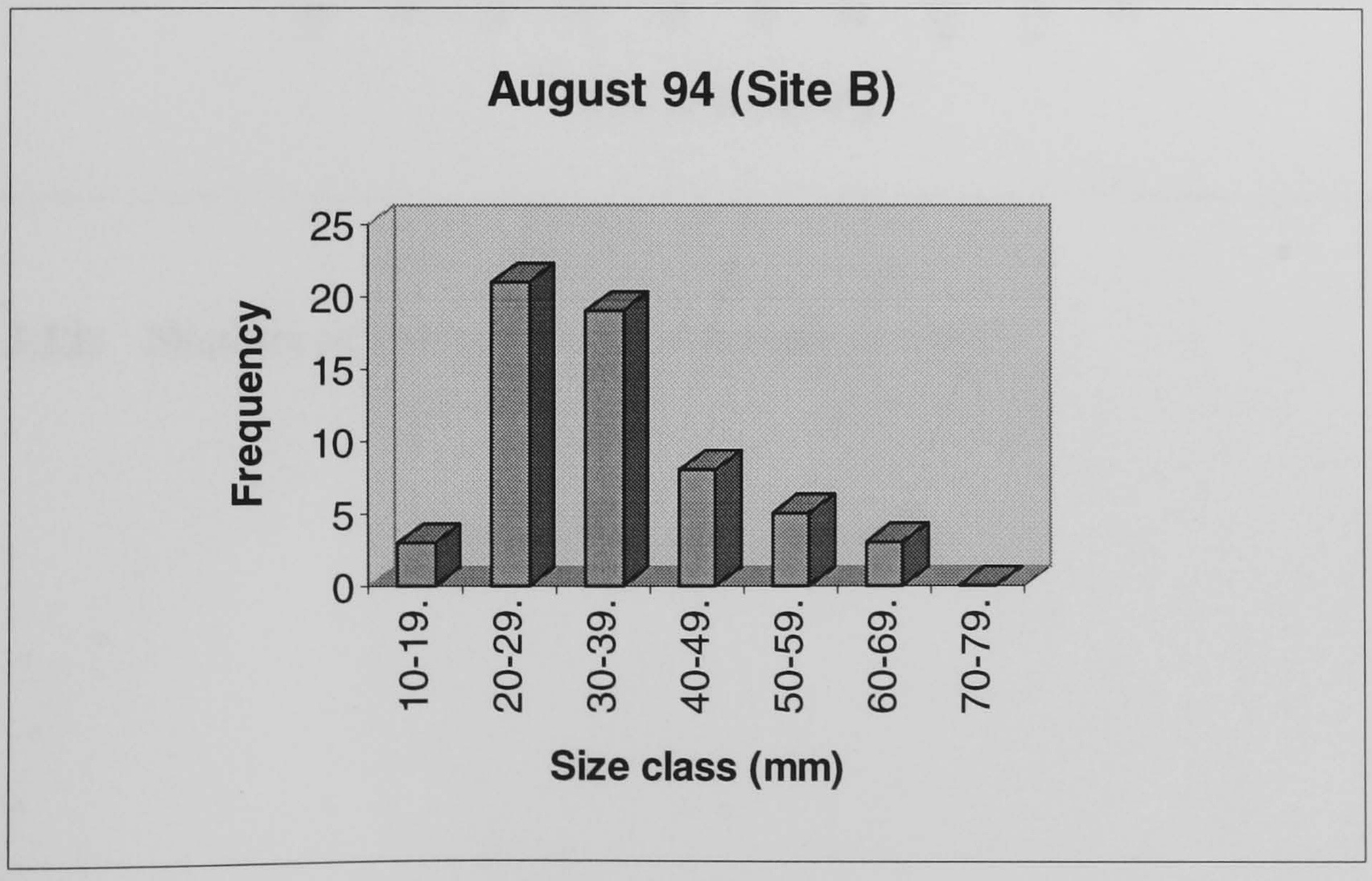


Figure 3.11: Length - frequency composition for the August 1994 stickleback sample from site B.

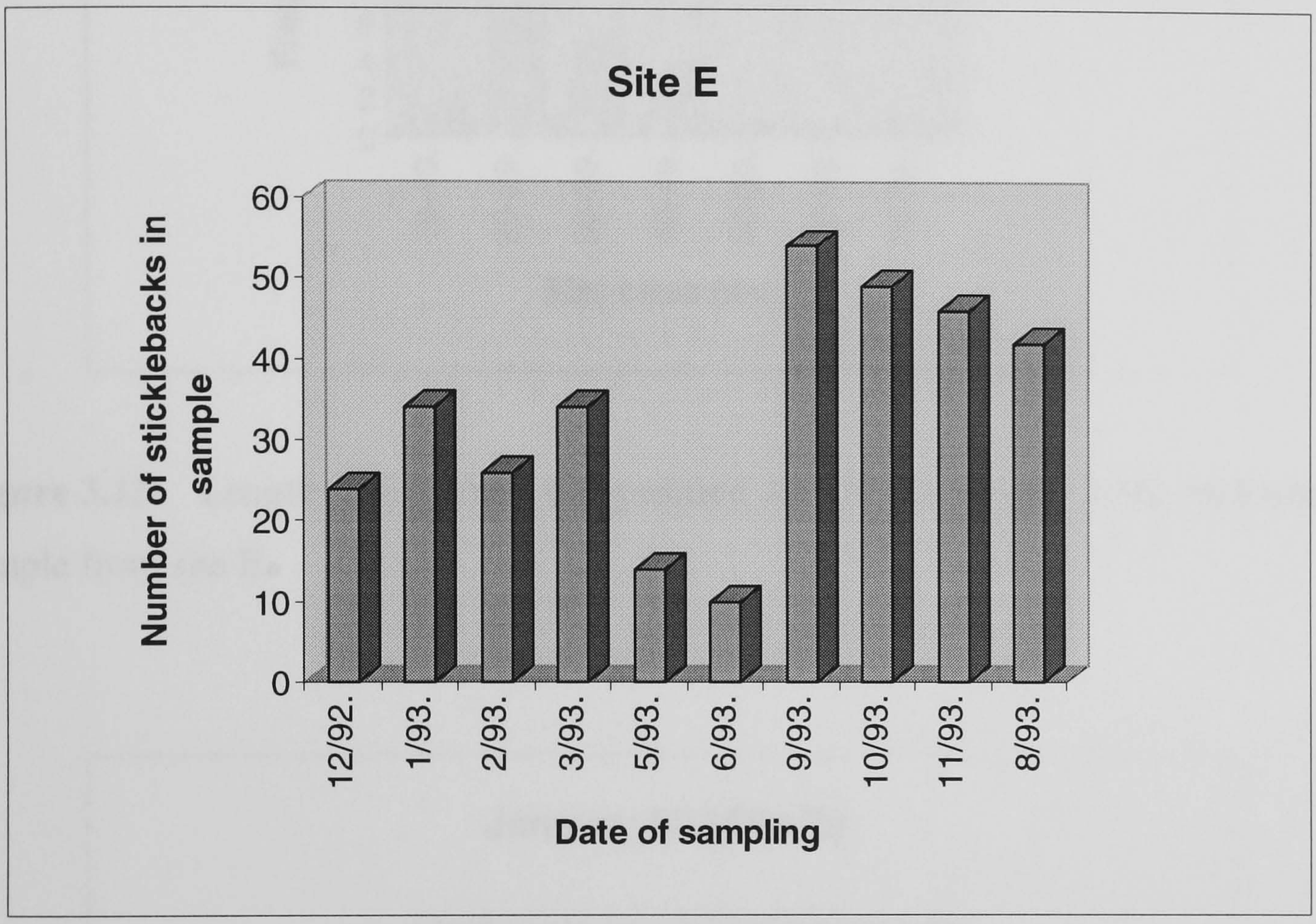


Figure 3.12: Number of fish per seasonal sample at site E.

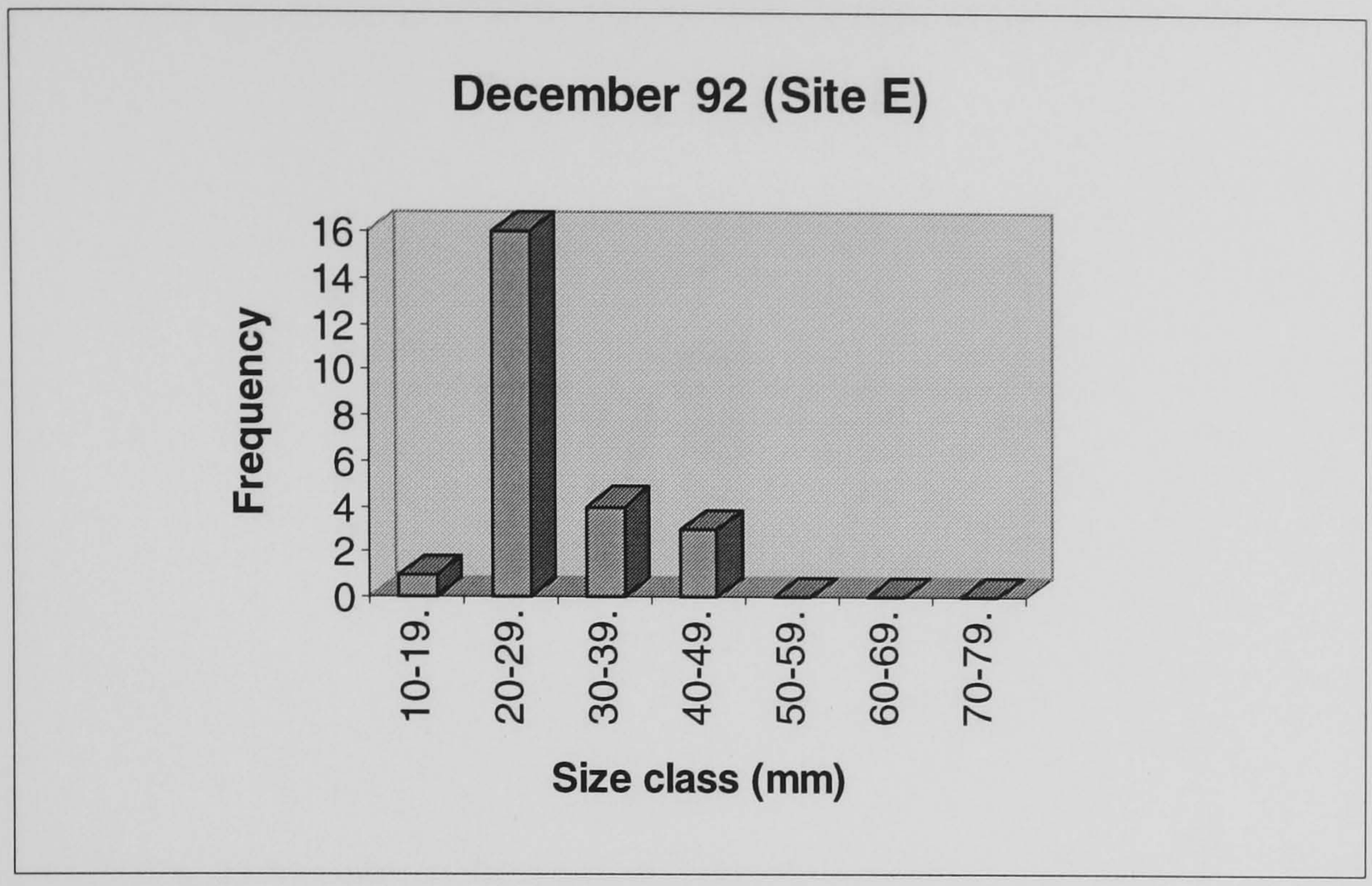


Figure 3.13: Length - frequency composition for the December 1992 stickleback sample from site E.

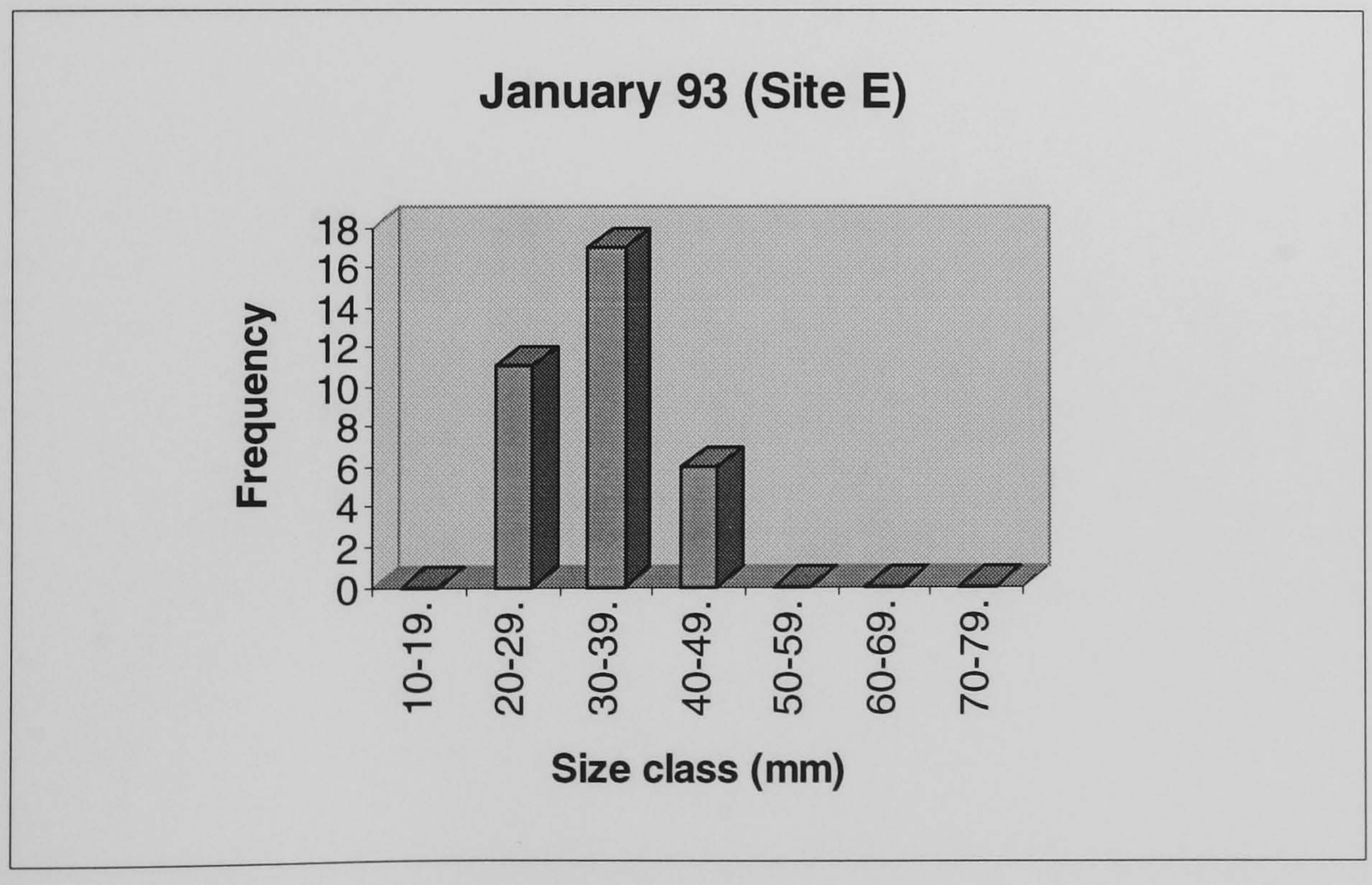


Figure 3.14: Length - frequency composition for the January 1993 stickleback sample from site E.

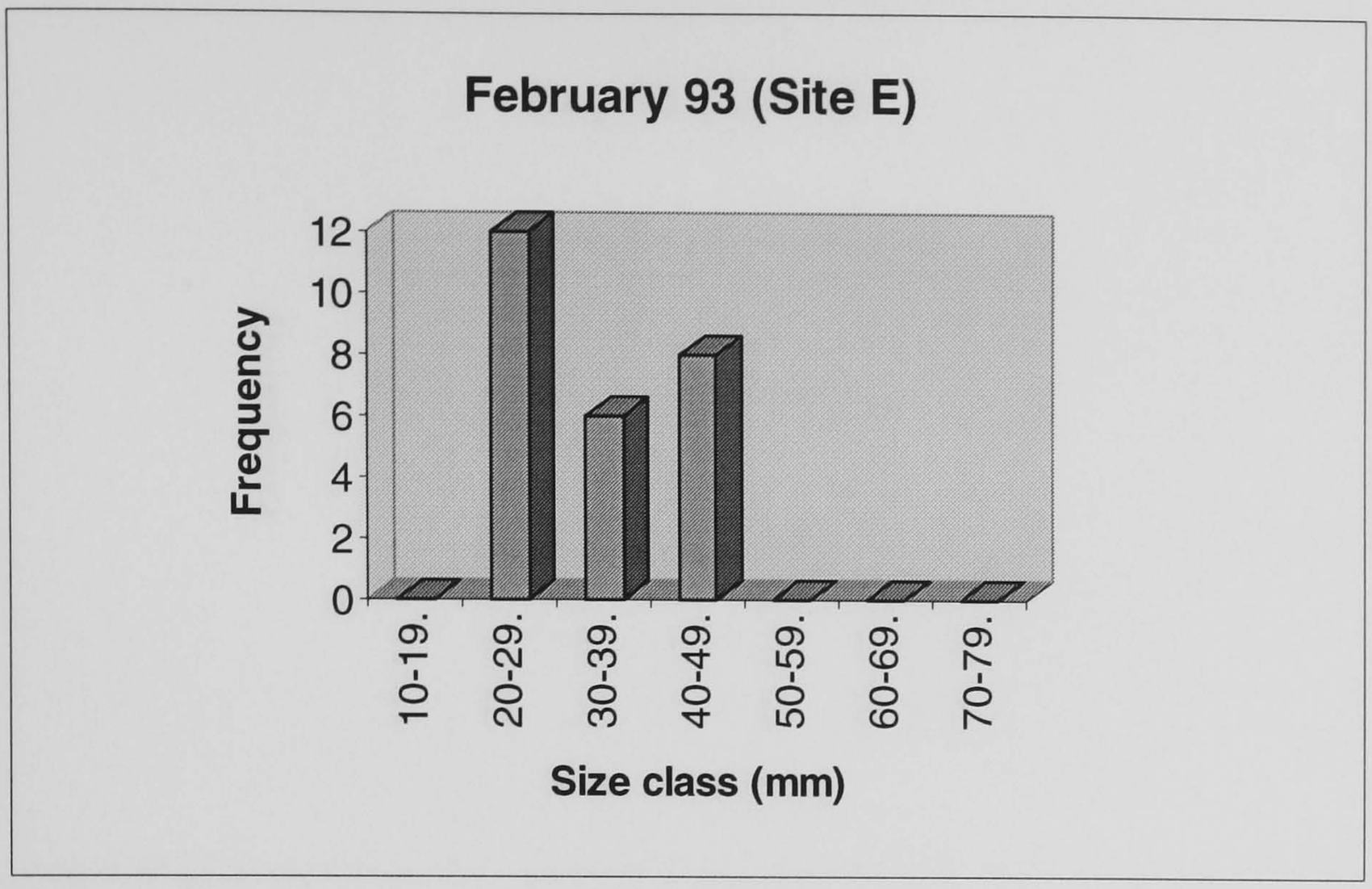


Figure 3.15: Length - frequency composition for the February 1993 stickleback sample from site E.

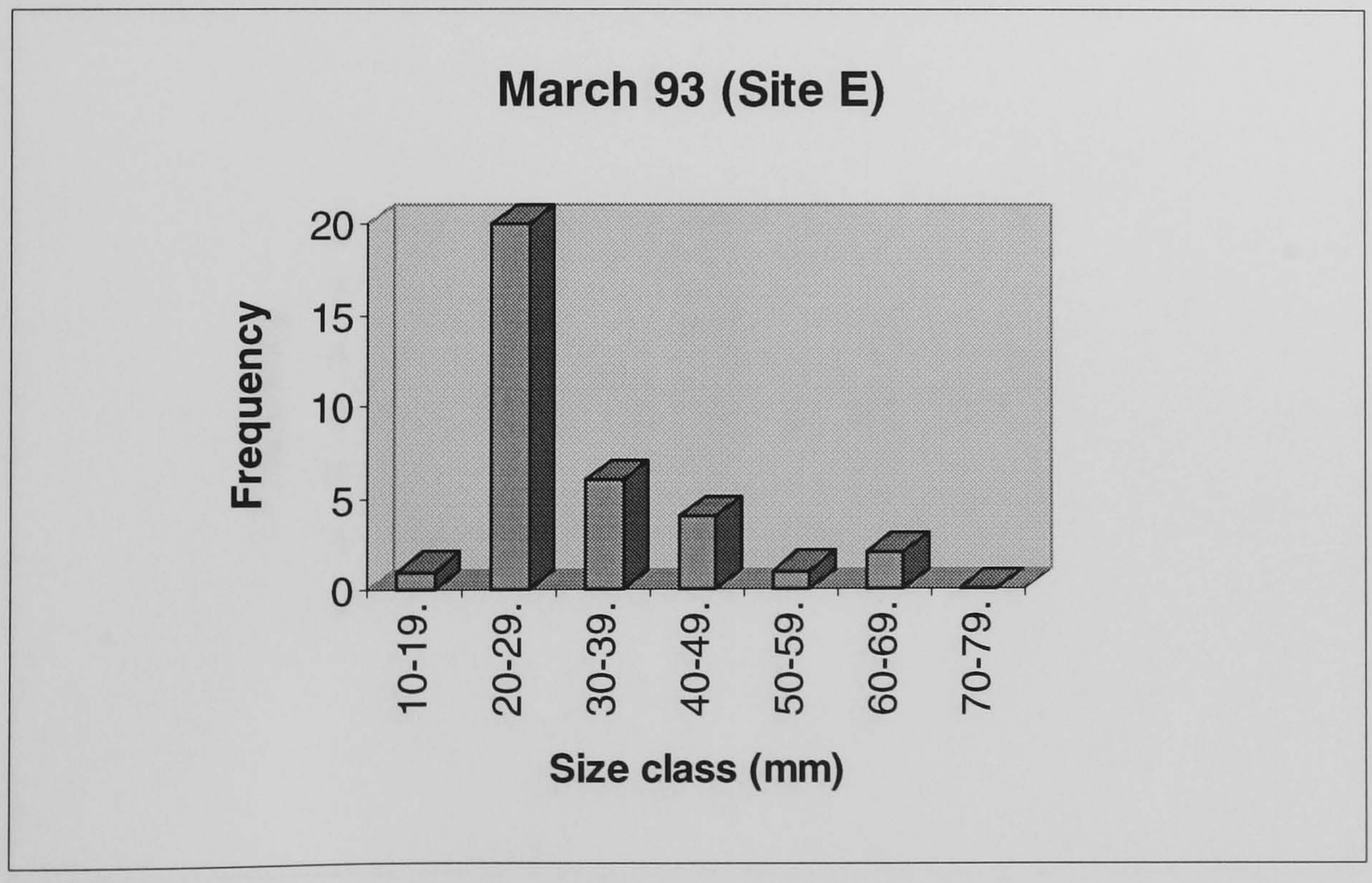


Figure 3.16: Length - frequency composition for the March 1993 stickleback sample from site E.

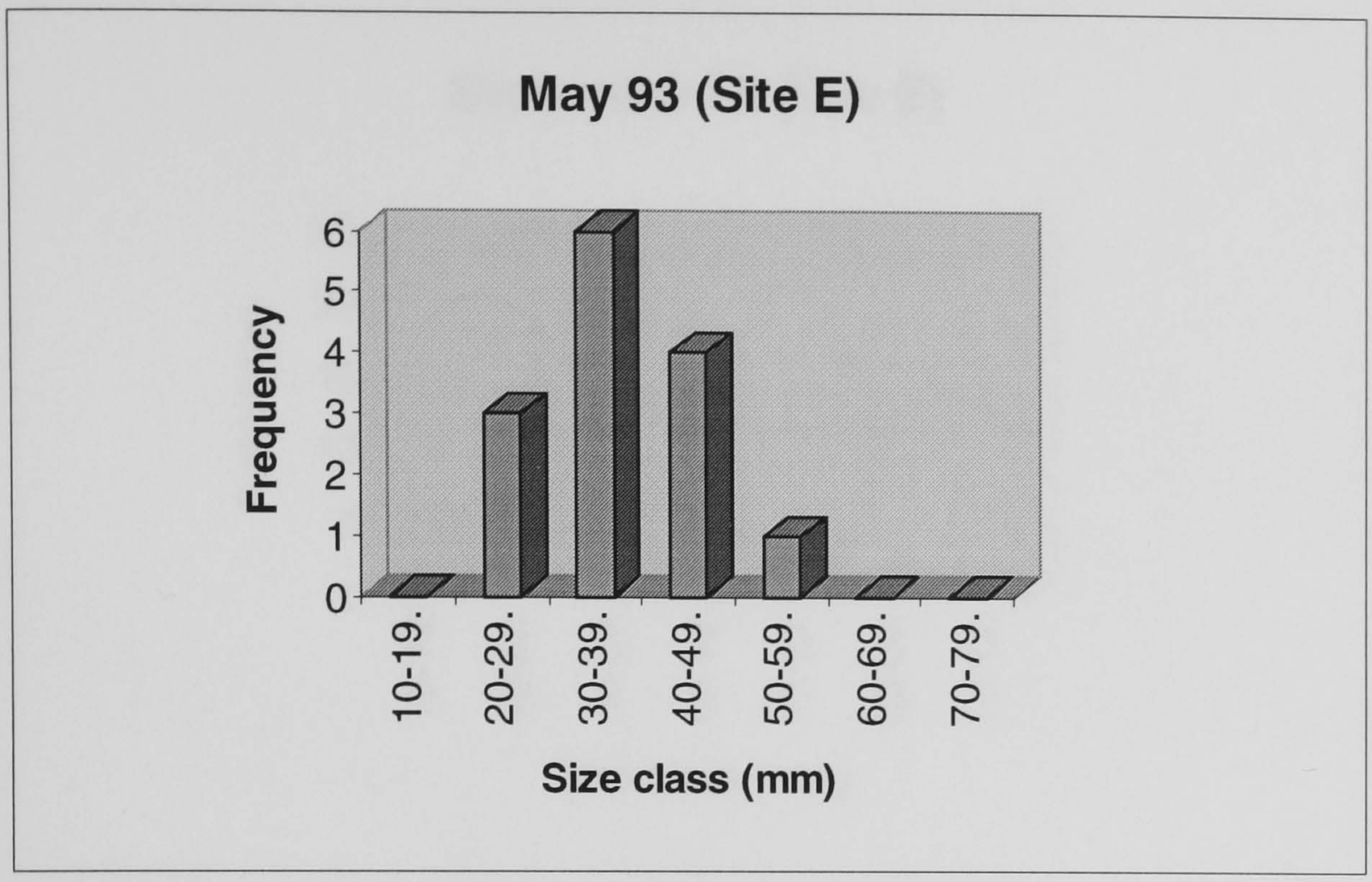


Figure 3.17: Length - frequency composition for the May 1993 stickleback sample from site E.

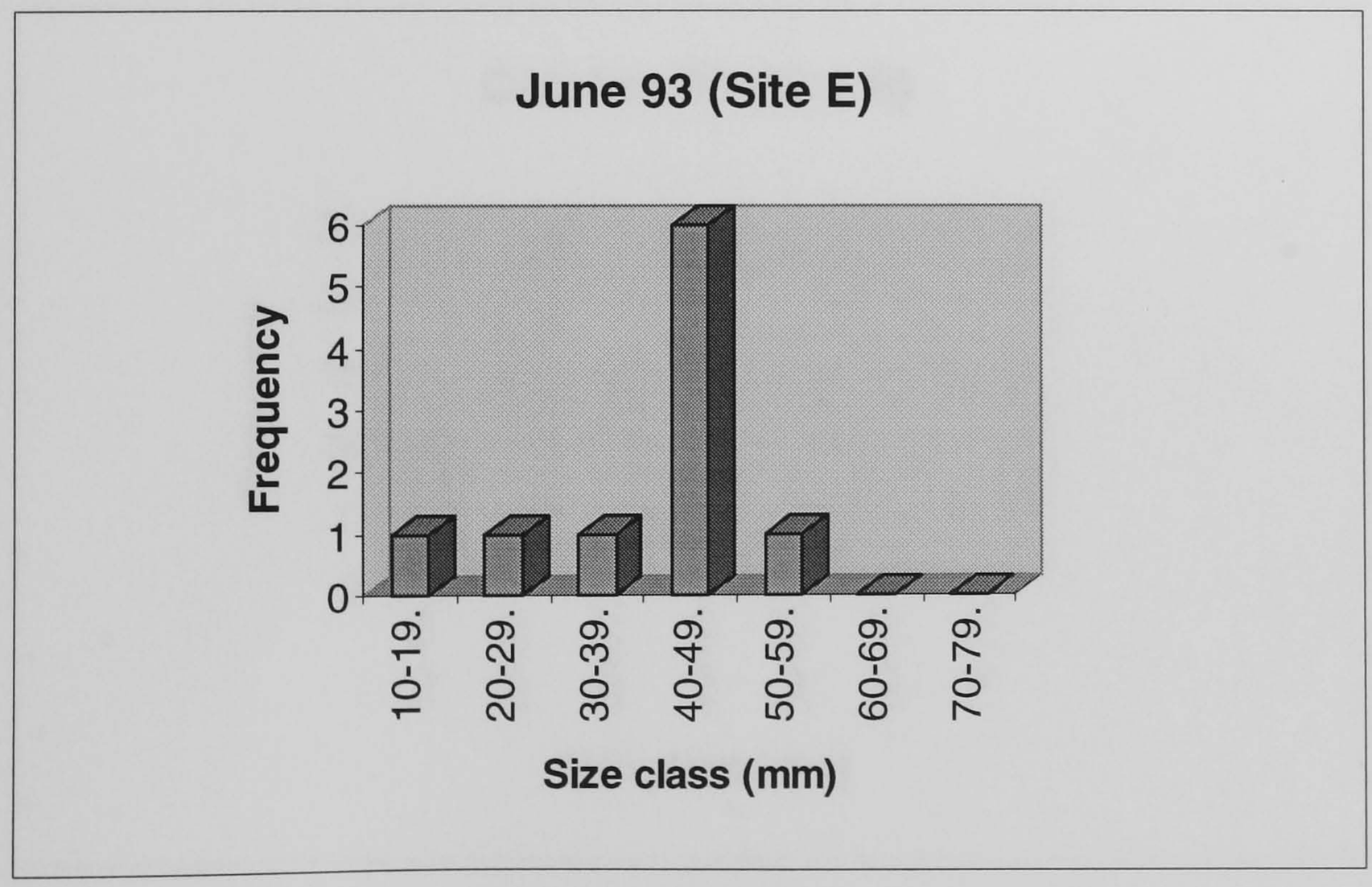


Figure 3.18: Length - frequency composition for the June 1993 stickleback sample from site E.

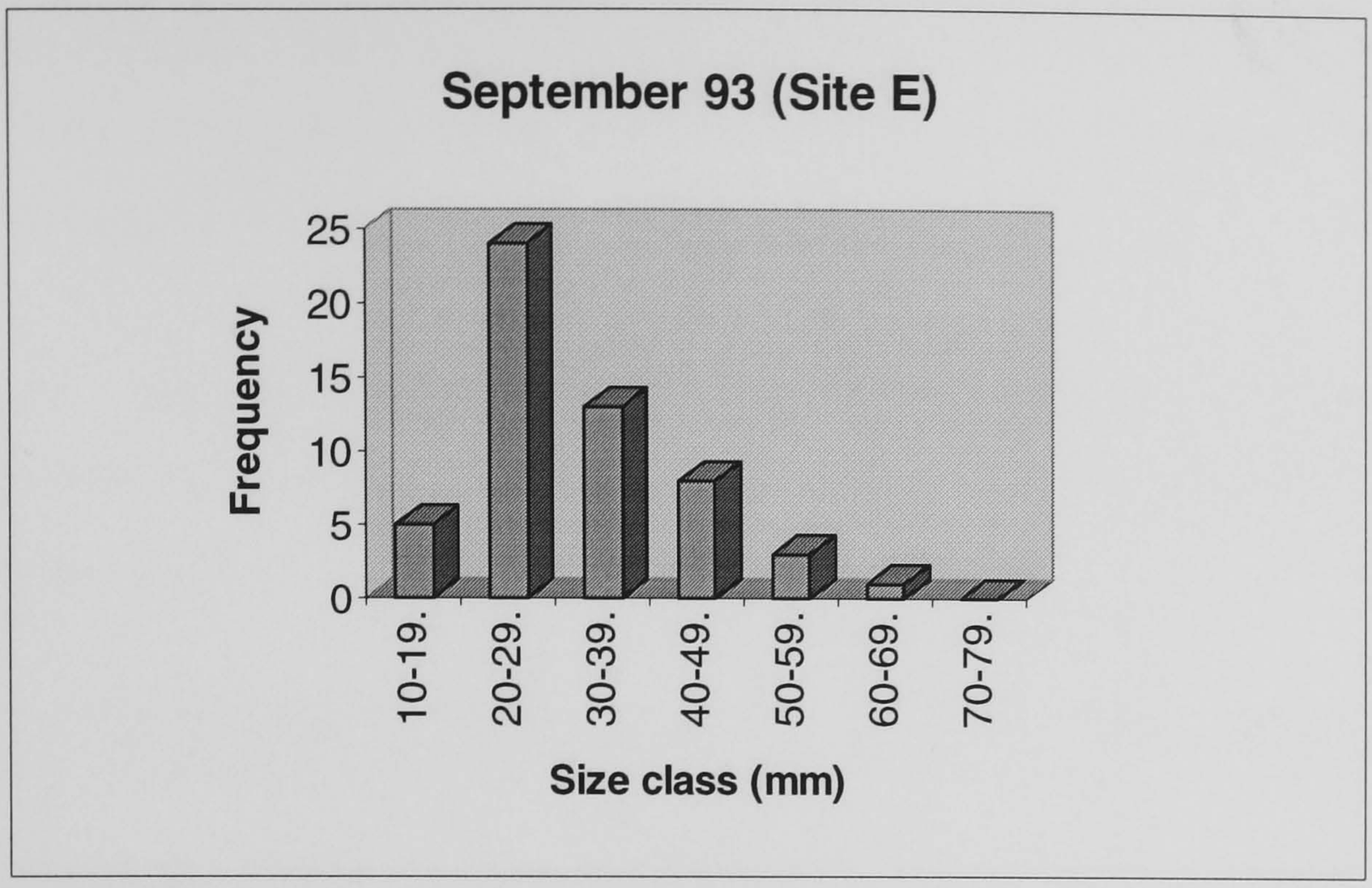


Figure 3.19: Length - frequency composition for the September 1993 stickleback sample from site E.

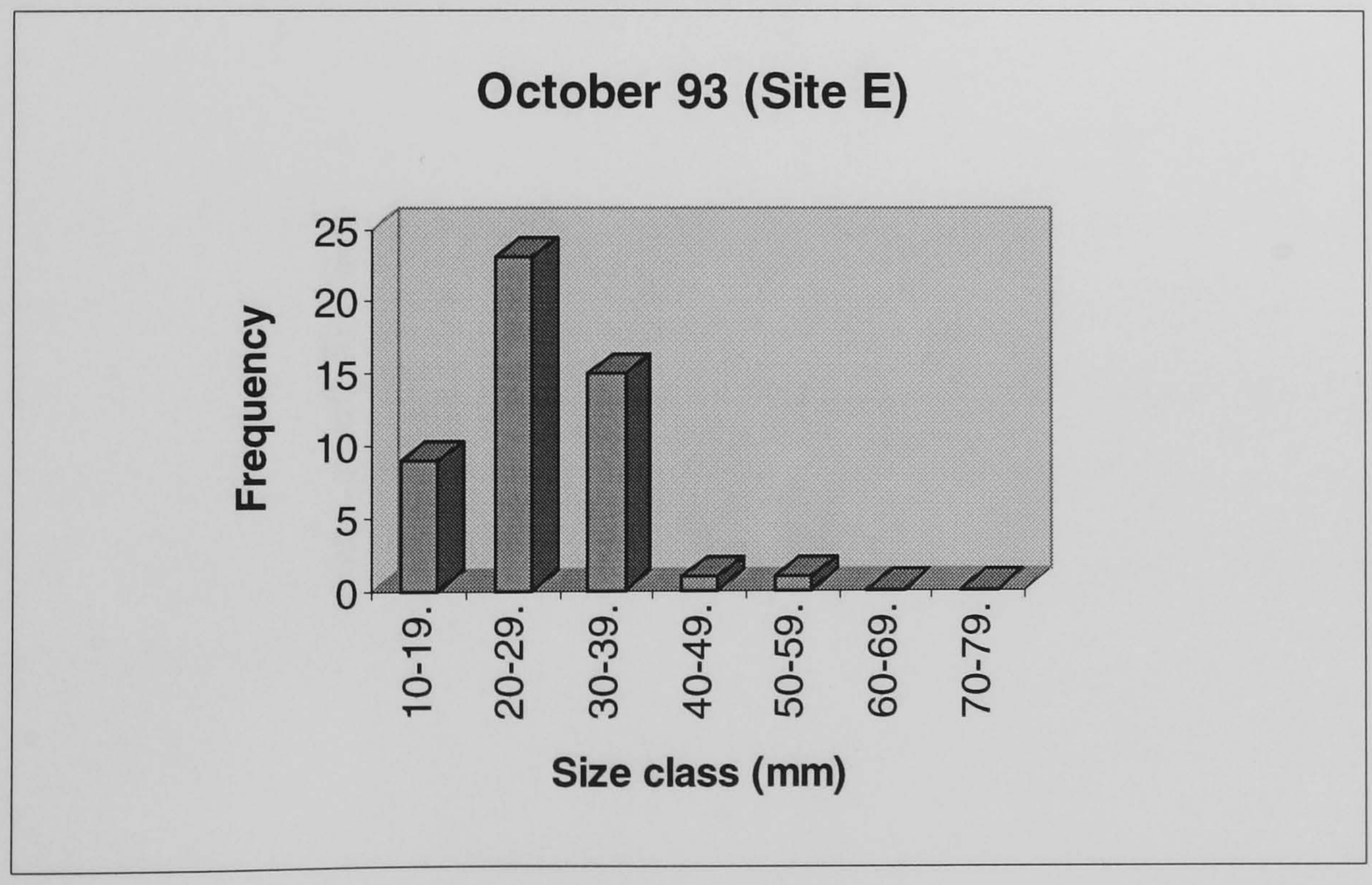


Figure 3.20: Length - frequency composition for the October 1993 stickleback sample from site E.

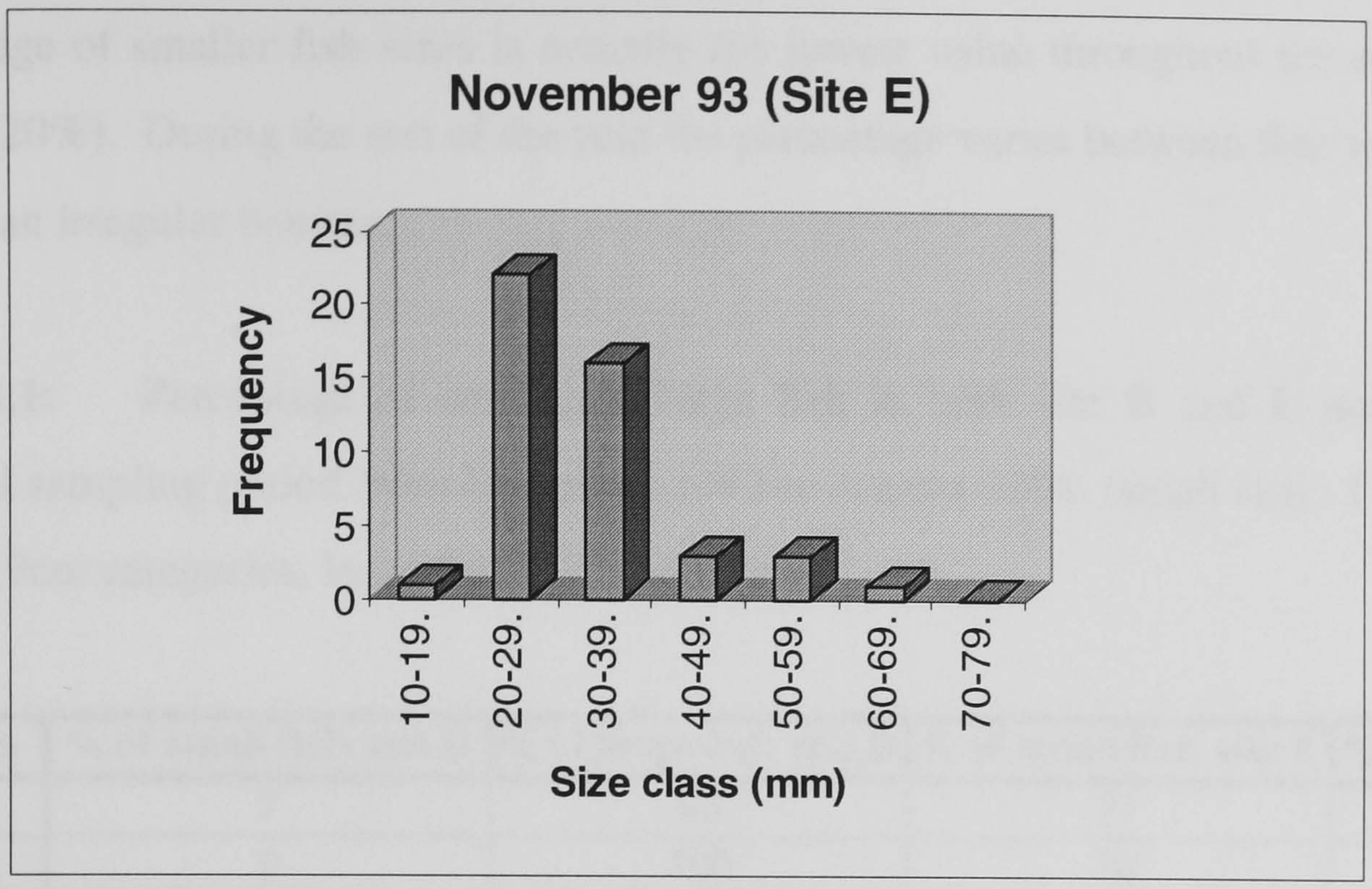


Figure 3.21: Length - frequency composition for the November 1993 stickleback sample from site E.

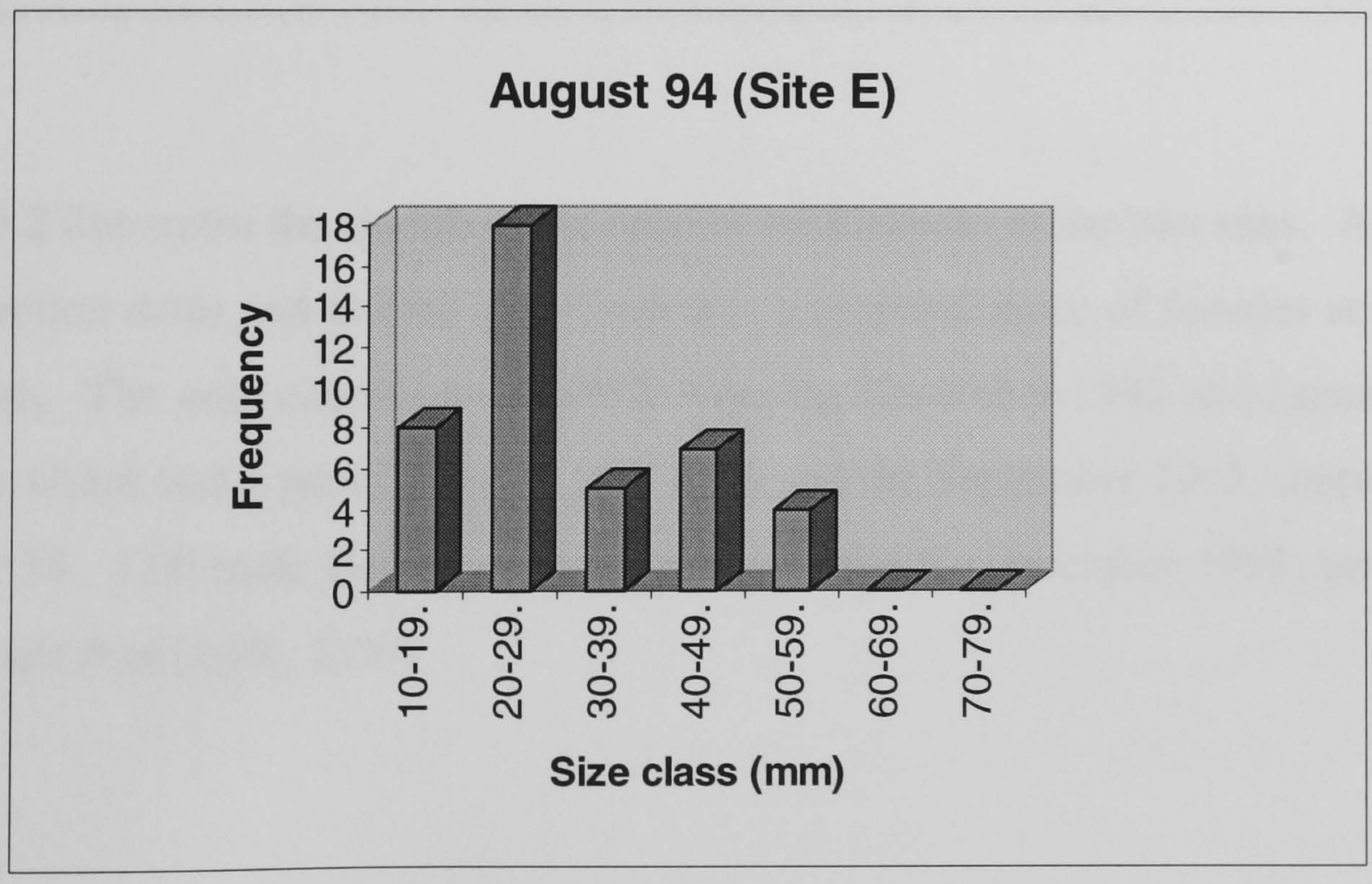


Figure 3.22: Length - frequency composition for the August 1994 stickleback sample from site E.

No such pattern is clearly apparent at site E (see **Table 3.1**). In contrast, the June percentage of smaller fish sizes is actually the lowest value throughout the sampling period (20%). During the rest of the year the percentage varies between that value and 71% in an irregular manner.

Table 3.1: Percentage of small and large fish at both site B and E during the seasonal sampling period from December 1992 to August 1994. (small fish : 1 - 1.9cm and 2-2.9cm categories, large fish 3cm and larger).

Months	% of small fish site B	% of large fish site B	% of small fish site E	% of large fish site E
12.92	7	93	71	29
1.93	0	100	32	68
2.93	0	100	46	54
3.93	0	100	62	38
5.93	0	100	21	79
6.93	82	18	20	80
9.93	67	33	54	46
10.93	38	62	65	35
11.93	35	65	50	50
8.94	41	59	62	38

Table 3.2 illustrates the changing sex ratio of sticklebacks at the two sites. At almost all collection dates and at both sites there was a preponderance of females among the adult fish. The only exceptions at site E were the December 1992 and January 1993 samples which had a parity value (1.00 : 1.00) and the September 1993 sample which was a 1.35 : 1.00 male biased. One collection at site B (December 1992) provided a slight male bias (1.08 : 1.00).

Table 3.2: Sexual composition of Wandle sticklebacks sampled from both sites B and E.

Sample date	Sex ratio site B Male : Female	Sex ratio Site E Male : Female
Dec-92	1.08:1.00	1.00:1.00
Jan-93	0.45:1.00	1.00:1.00
Feb-93	0.67:1.00	0.24:1.00
Mar-93	0.50:1.00	0.26:1.00
May-93	0.91:1.00	0.20:1.00
Jun-93	0.12:1.00	0.67:1.00
Sep-93	0.71:1.00	1.35:1.00
Oct-93	0.88:1.00	0.36:1.00
Nov-93	0.64:1.00	0.77:1.00
Aug-94	0.51:1.00	0.17:1.00

3.3.2. The Parasite Fauna

Five species of parasites were recovered from sticklebacks during the present study; namely *Schistocephalus solidus* (Muller, 1776), *Trichodina megamicronucleata* (Hirschmann and Partsch, 1955), *Gyrodactylus arcuatus* (Bykhovskii, 1933), *Glugea anomala* (Moniez, 1887), and *Proteocephalus filicollis* (Rudolphi, 1802).

As mentioned earlier (see section 3.1) the only previous parasitiological study involving sticklebacks as hosts in the River Wandle is that of Chen (1992). In her study most emphasis was placed on data relating to *S. solidus*. As Chen's study only concerned parasite analysis based on the gross, external appearance of fish, it was unable to identify infections with *Trichodina* or *Gyrodactylus* (both of microscopic size), or with cysts like *Glugea* or with *Proteocephalus* (parasite within the gut), nor was it able to quantify the levels of intensity of *S. solidus* infection.

Preliminary samples taken at 10 different sites in the River Wandle revealed that *S. solidus* infections were present in sticklebacks at most of them. Subsequent detailed

collections and analysis have been restricted to two sites (B and E) characterised by initial high and low levels of infection respectively (**Table 3.3**).

Table 3.3 describes the prevalence and the mean intensity ranges of the five parasites found in *G. aculeatus* at sites B and E.

Table 3.3: Prevalence and mean intensity ranges of parasites for monthly samples at each of the two collection sites between December 1992 and August 1994.

Parasites	Prevalence site B	Mean intensity site B	Prevalence site E	Mean intensity site E
S. solidus	22.7 - 100	0.48 - 4.7	4.08 - 42.9	0.05 - 0.6
T. megamicronucleata	100	-	100	-
G. arcuatus	22 -100	0.4 - 28.5	38.1 - 100	1.2 - 25.4
G. anomala	2.2 -15.3	0 - 0.2	7.1 - 70.8	0.07 - 0.7
P. filicollis	3.9 - 53.5	0.1 - 5.7	2.6 - 50.5	0 - 1

3.3.2.1. *Schistocephalus solidus*

INTRODUCTION

Chapter 1 (see section 1.3.3) provides a description of the parasitological literature on *S. solidus* infections in fish.

Figure 3.23 shows uninfected three-spined sticklebacks *G. aculeatus* from the River Wandle. **Figure 3.24** and **Figure 3.25** illustrate abdominal distention associated with infection by the plerocercoids of *S. solidus*.

RESULTS

Prevalence, intensity and mean intensity patterns

Consideration was made of the nature of the parasite infection at both sites B and E. Site B was considered to be the heavily - parasitised site whereas, conversely, site E showed a lesser degree of parasitic infection. Quantitative analyses were performed of the prevalence, intensity, mean intensity, parasitic indices and condition factor using established methods for calculating these (Arme and Owen, 1967 and Pennycuick, 1971), and site- specific differences were noted.

Consistently high levels of parasite prevalence were seen at site B (**Figure 3.26**). From December 1992 to May 1993 the prevalence of *S. solidus* infection was 100%. From June 1993 - November 1993 the prevalence of infection declined to values between 22.7 - 57.4%.

The prevalence for August 1994 was 40% so it is not possible to say whether or not the change in mid - 1993 was part of a cyclical pattern or a more permanent alteration. The change to lower prevalence levels between May and June 1993 did however correspond in time with the increase on the proportion of smaller, juvenile fish at site B.

Parasite intensities and mean intensities at site B reflected the general pattern shown by the prevalence results. During the months from December 1992 to May 1993 the parasitisation level varied between 2.7 and 4.7 parasites per fish in terms of both intensity and mean intensity. This pattern was seen to change, however, for the latter

Figure 3.23: Two typical examples of uninfected Wandle sticklebacks.

Figure 3.24: Four three - spined sticklebacks showing conspicuous abdominal distention associated with infection by plerocercoids of *S. solidus*.

Figure 3.25: Dissection of the peritoneal cavity of a stickleback to reveal portions of two *S. solidus* plerocercoids. Two additional plerocercoids from another fish have been added to the illustration to show the size and overall configuration of these parasites.

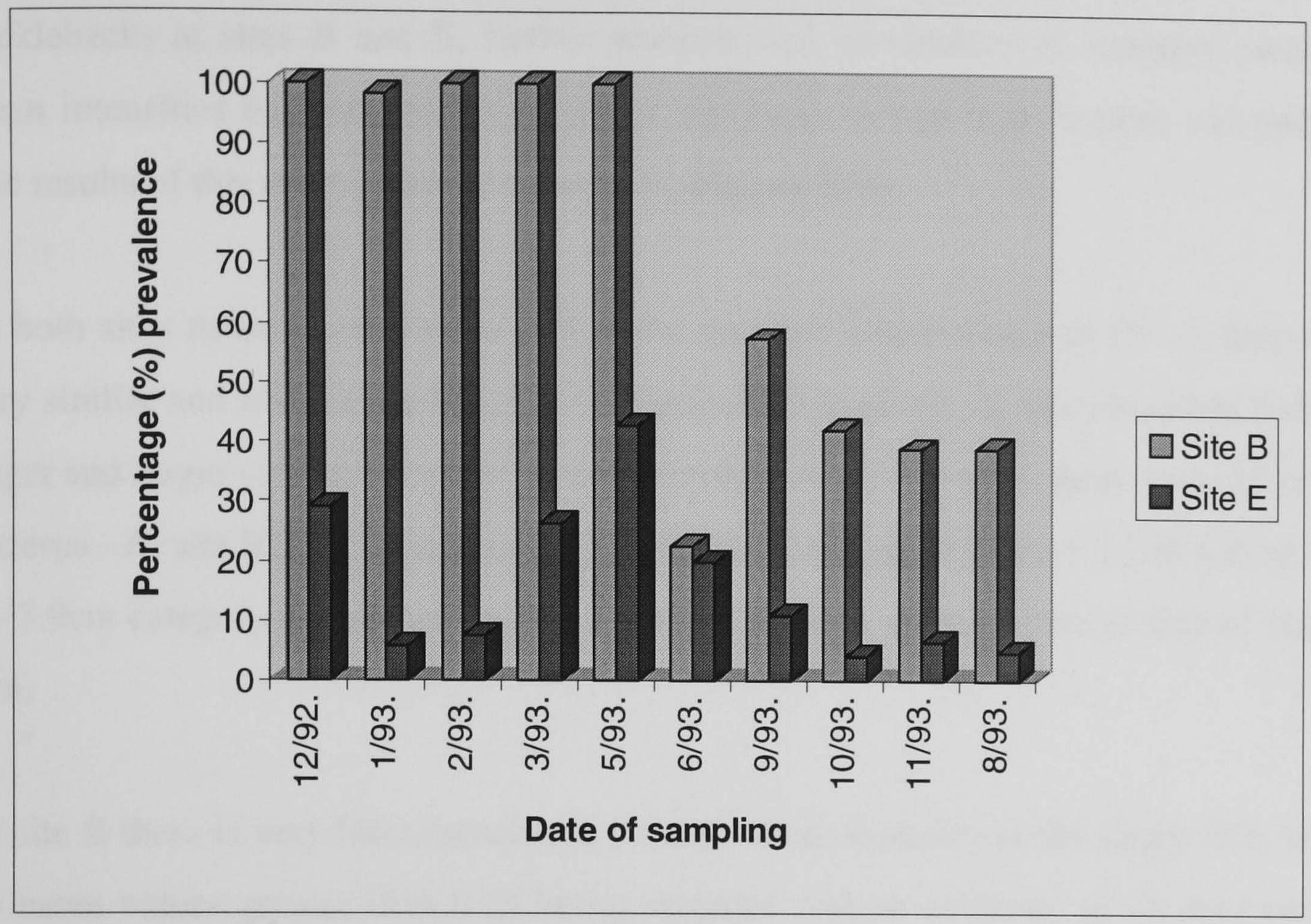


Figure 3.26: Prevalence of *Schistocephalus solidus* infection in *Gasterosteus aculeatus* at site B and E on the River Wandle.

part of the sampling programme with mean intensities, were markedly lower at site B, in the range 0.48 - 0.91 parasites per fish (**Figure 3.27**).

Site E was wholly contrasting with respect to site B in terms of prevalence, intensity and mean intensity. Typically, the prevalence of parasitism was very much less than that of site B. The highest prevalence recorded for site E was for May 1993, with a prevalence of 42.9% and the lowest being for October 1993 at 4.1%. Unlike the data for site B, the picture at site E did show any marked reduction in prevalence from June 1993 onwards.

Intensity of parasitism at site E was always low. In terms of intensity, this value was between 1.0 - 2.1 parasites per fish. Mean intensity showed a greater degree of variation with values ranging from 0.04 - 0.64 parasites per fish (**Figure 3.28**).

Because previous data had shown that there were differences in the sizes of sticklebacks at sites B and E, further analysis was undertaken to compare parasite mean intensities between the two sites on the basis of fish size (length) categories. The results of this analysis are illustrated in (**Figure 3.29**).

At both sites mean intensities in fish in the smallest length category (1 - 1.9cm) are very similar and low, being below 0.1. Thereafter, however, as one considers fish of larger and larger size, the parasite mean intensities at the two sites show very different patterns. At site B, the mean intensity rapidly rises to a level of over 2.0 in fish in the 3 - 3.9cm category and thereafter remains at levels at or above 2.0 in all fish of larger size.

At site E there is very little increase in parasite mean intensity in the larger fish, with no mean values greater than 0.29 being recorded and no evidence of an increase in mean intensity with host size being obvious.

Parasite population structure

When weight class frequencies of *S. solidus* plerocercoids are examined (see **Figure 3.30 - 3.39** for site B and **Figure 3.40 - 3.49** for site E) it is clear that there are differences in the pattern of weight class frequencies at the two sites. **Figure 3.50** compares the mean weight class frequencies for the two sites but the differences in parasite weights are perhaps most clearly demonstrated in **Figure 3.51** which compares the mean total parasite weight per fish from sites B and E for each of the sample dates. Examination of this figure shows that for all monthly samples apart from June 1993 and September 1993 the mean weight of parasites from site B is greater than that at site E.

As the intensities of parasitization at sites B and E were so different, it was decided to perform an analysis which considered the possible impact of parasite density within individual fish on the mean weight of parasites in that fish. This analysis was

performed to test the hypothesis that higher parasite densities at site B might result in smaller mean parasite weights.

The analysis was carried out in a number of ways. First, three monthly samples from site B (December 1992, January 1993 and February 1993) were examined on the basis of individual fish with one or more plerocercoids. Results are summarised in **Figures 3.52**, **3.53** and **3.54** in each case with a best fit logarithmic curve drawn through the individual data points. In each case, the mean parasite weight clearly declined with increasing parasite density. So, for each of the months the mean parasite weight in fish containing only a single plerocercoid was between 200 and 300mg while in fish with a density of 6 or more the mean weight is less than 100mg. When, however, a summated analysis was made of all infected fish from site E (see **Figure 3.55**) a remarkable and unexpected result was apparent, as the mean parasite weight at density 1 was less than 100mg and declined further at higher densities.

Figure 3.56 illustrates this difference as a comparison between the summated sample from E and the January 1993 sample from site B.

Figure 3.57 is a compound graph of the individual fish data points at site B (December 1992, January 1993 and February 1993) and the summated site E data. It shows the close correspondance between the best fit lines for the site B samples and the much reduced values for site E.

A comparison of the mean weights of parsites at density one carried out between (Dec. 92 and Jan. 93), (Dec.92 and Feb. 93) and (Jan 93 and Feb. 93) at site B indicates there were no significant differences between these three samples while there was a very significant difference between the total sample at site E and the mean of the three samples at site B.

Table 3.3a: Means and standard errors of the parasite weight at density 1 for Dec. 92, Jan. 93, Feb. 93 and mean of these three samples at site B and all samples at site E.

	Dec. 92 site B	**Jan. 93 site B**	**Feb. 93 site B**	**Mean of three sample site B**	**All sample site E**
Mean	236.7	229.2	284.3	253.9	75.1
SE	24	20	25	14	15

Dec. 92 - Jan. 93	T = 0.24	p = 0.82	DF = 11
Dec. 92 - Feb. 93	T = -1.37	p = 0.19	DF = 14
Jan. 93 - Feb. 93	T = -1.72	p = 0.098	DF = 24
All three (site B) and all samples (site E)	T = 8.72	p = 0.0000	DF = 59

The conclusion from these investigations of the impact of parasite density on parasite size is that

(i) at both sites there is a density - dependent decline in size with increasing density, and

(ii) at site E the mean parasite size is much less than at site B, even when the effect of parasite density is accounted for.

Parasitic indices

Figure 3.58 illustrates the changing pattern of *S. solidus* parasitic indices in sticklebacks at sites B and E. Parasitic index is a complex and compound measure of infection taking into account (in infected fish alone) the summated biomass of plerocercoids (i.e. numbers and individual biomasses) in individual fish and the biomass of the host. Although complex, the index has the virtue of giving a value that probably mirrors the level of metabolic impact of the parasite burden on the host.

At both sites there was considerable variation in the parasitic index from month to month, although between December 1992 and May 1993 values at site B (varying between 44.1% and 25.6%) were consistently higher than those at site E (varying between 30.9% and 7.1%). Thereafter, values at the two sites were variable but overlapping, with those at site B declining to be similar to those at site E.

The parasitic index was found not to vary with intensity or prevalence at either site and it was difficult to identify any influence of seasonality on parasitic index at site E.

Figure 3.27: Intensity and mean intensity of *S. solidus* infection in *G. aculeatus* from the River Wandle at site B.

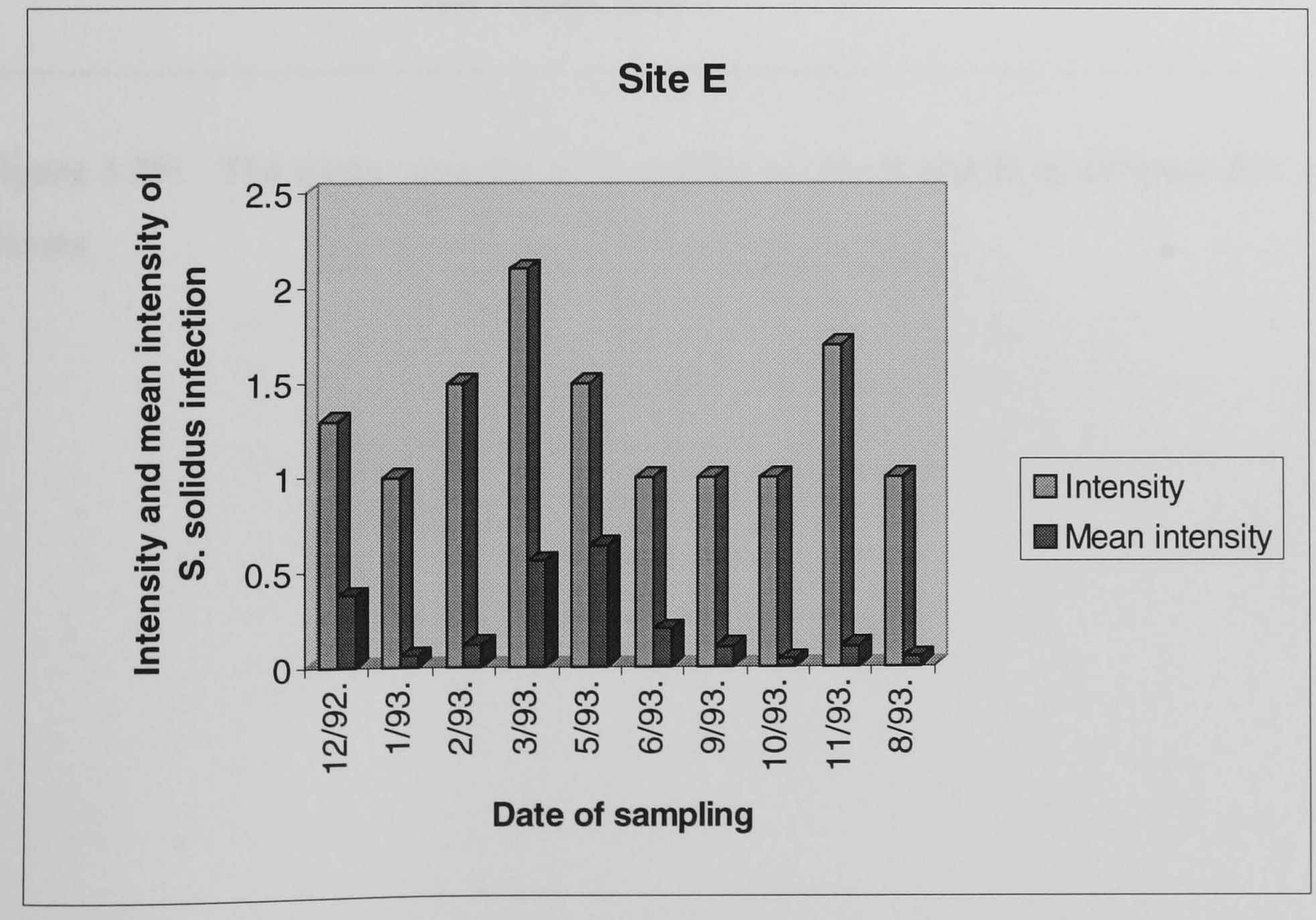


Figure 3.28: Intensity and mean intensity of *S. solidus* infection in *G. aculeatus* from the River Wandle at site E.

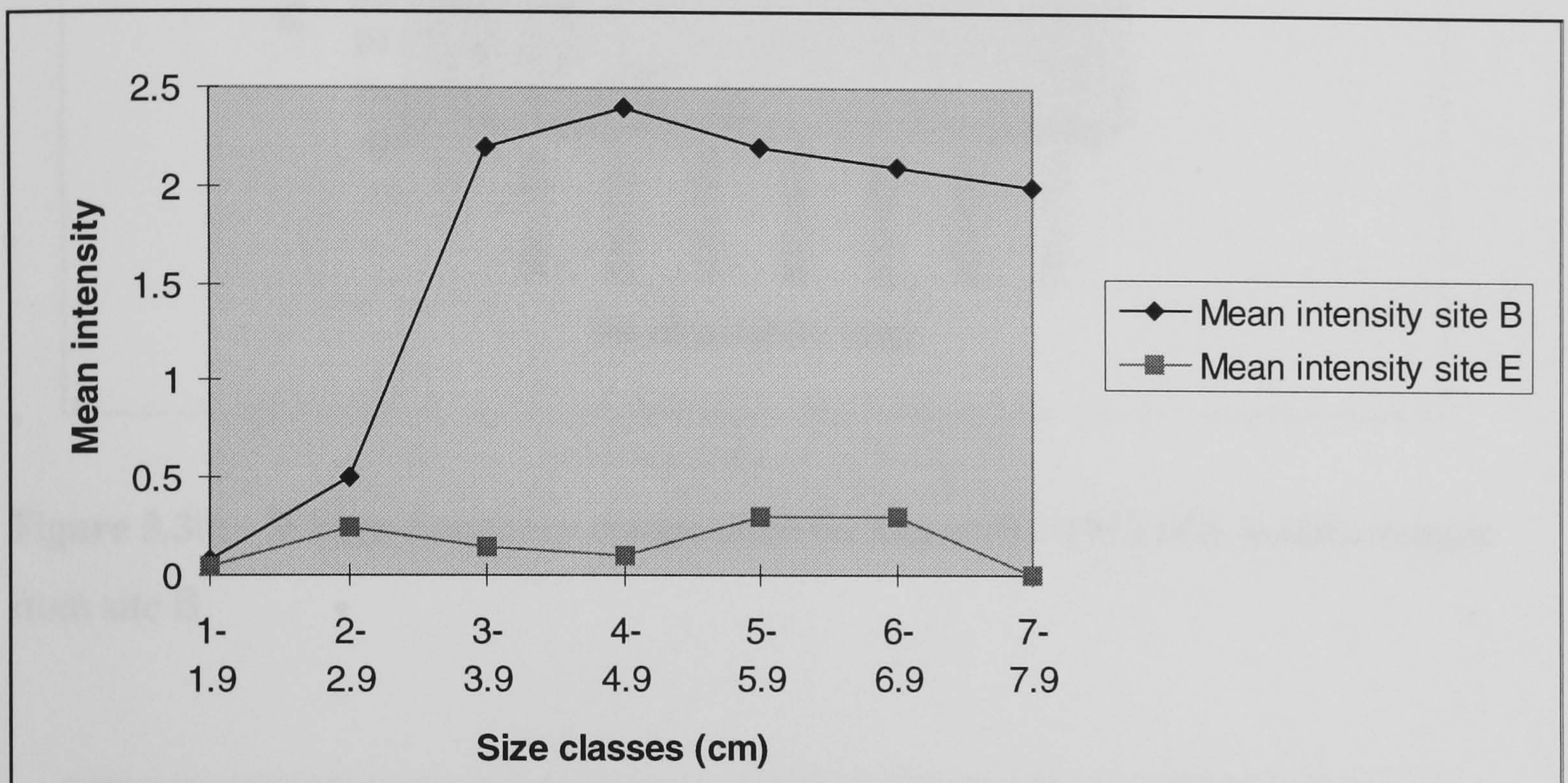


Figure 3.29: The mean intensity of *S. solidus* at site B and E in different fish size classes.

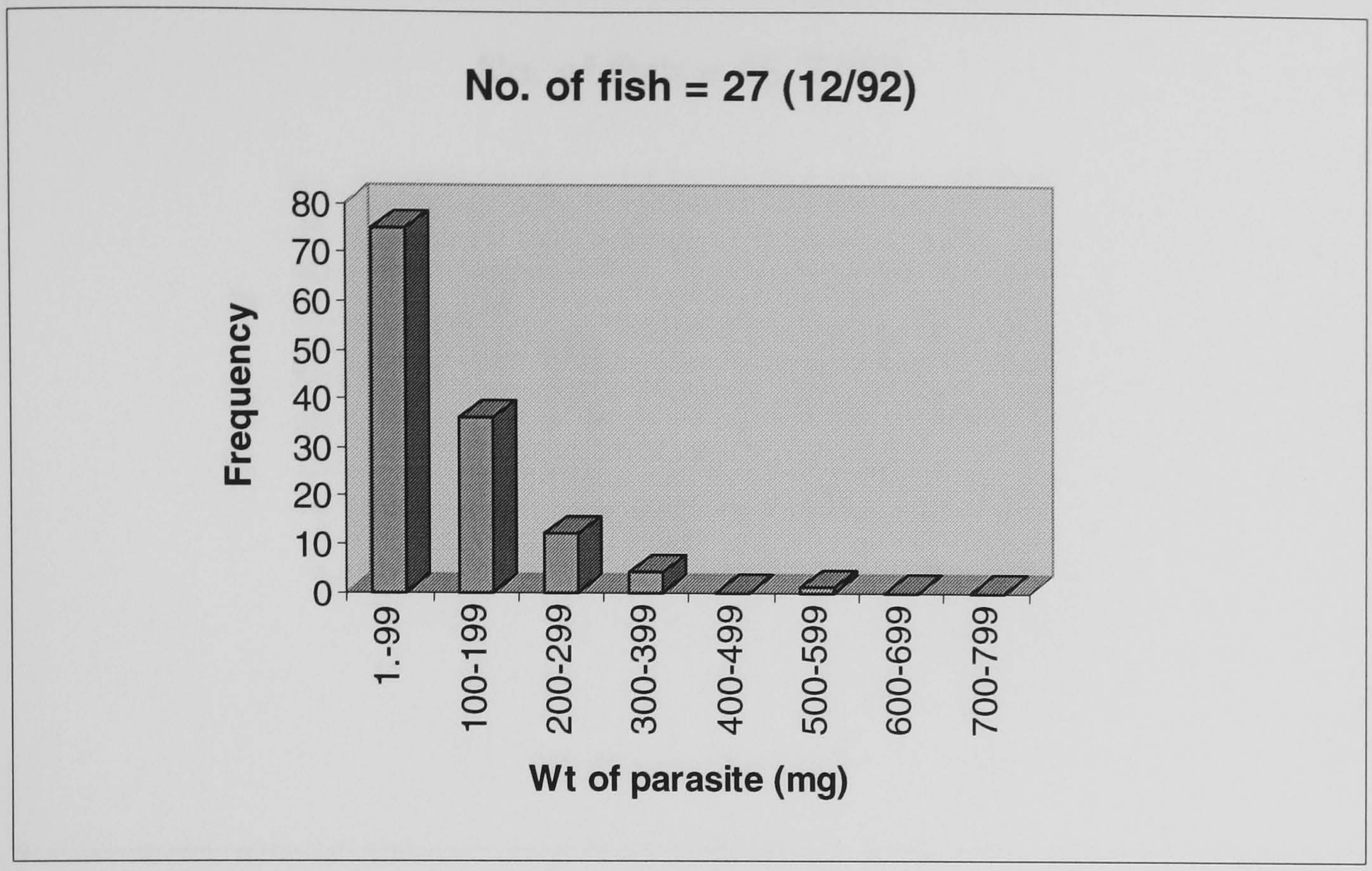


Figure 3.30: Weight frequency composition for December 1992 of *S. solidus* sample from site B.

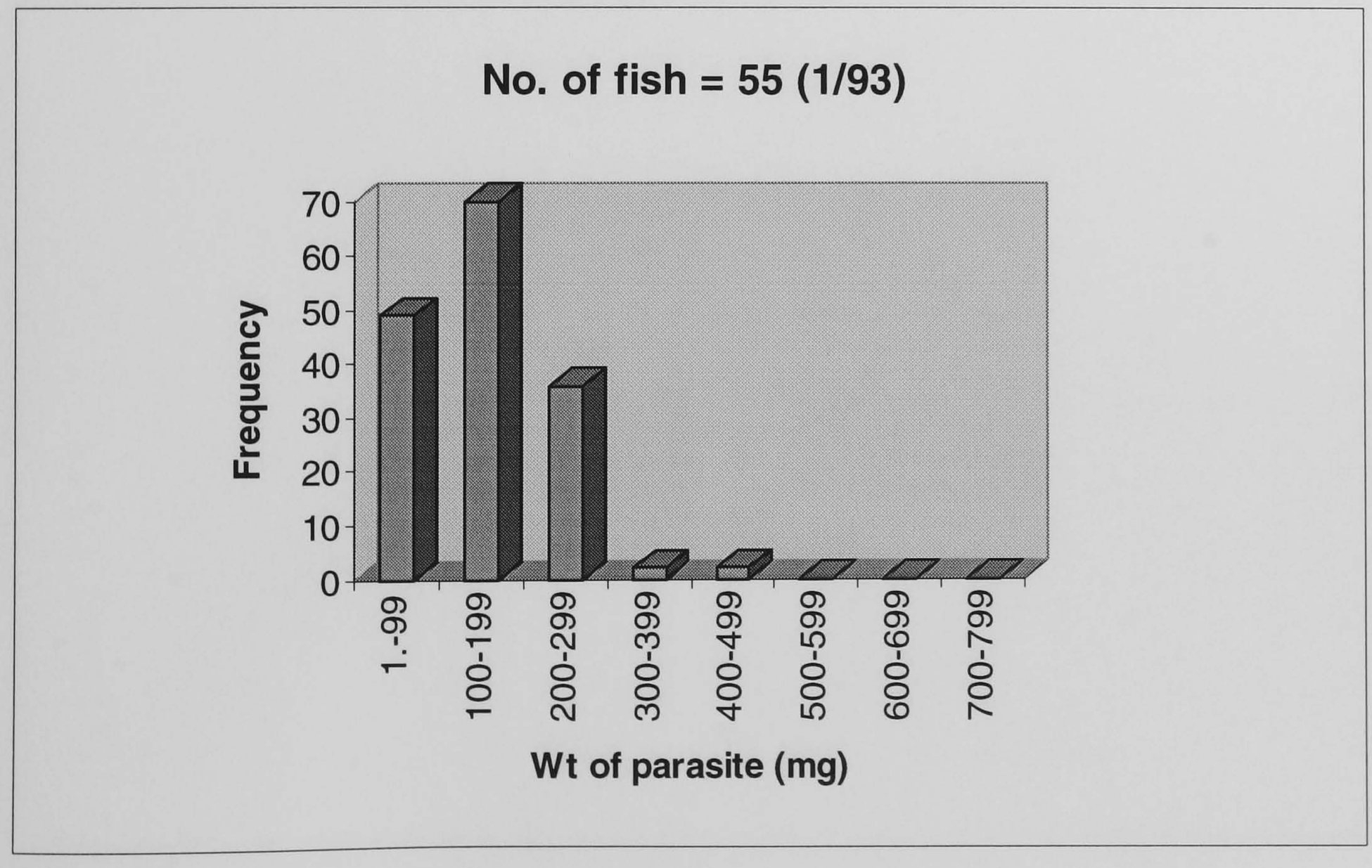


Figure 3.31: Weight frequency composition for January 1993 of *S. solidus* sample from site B.

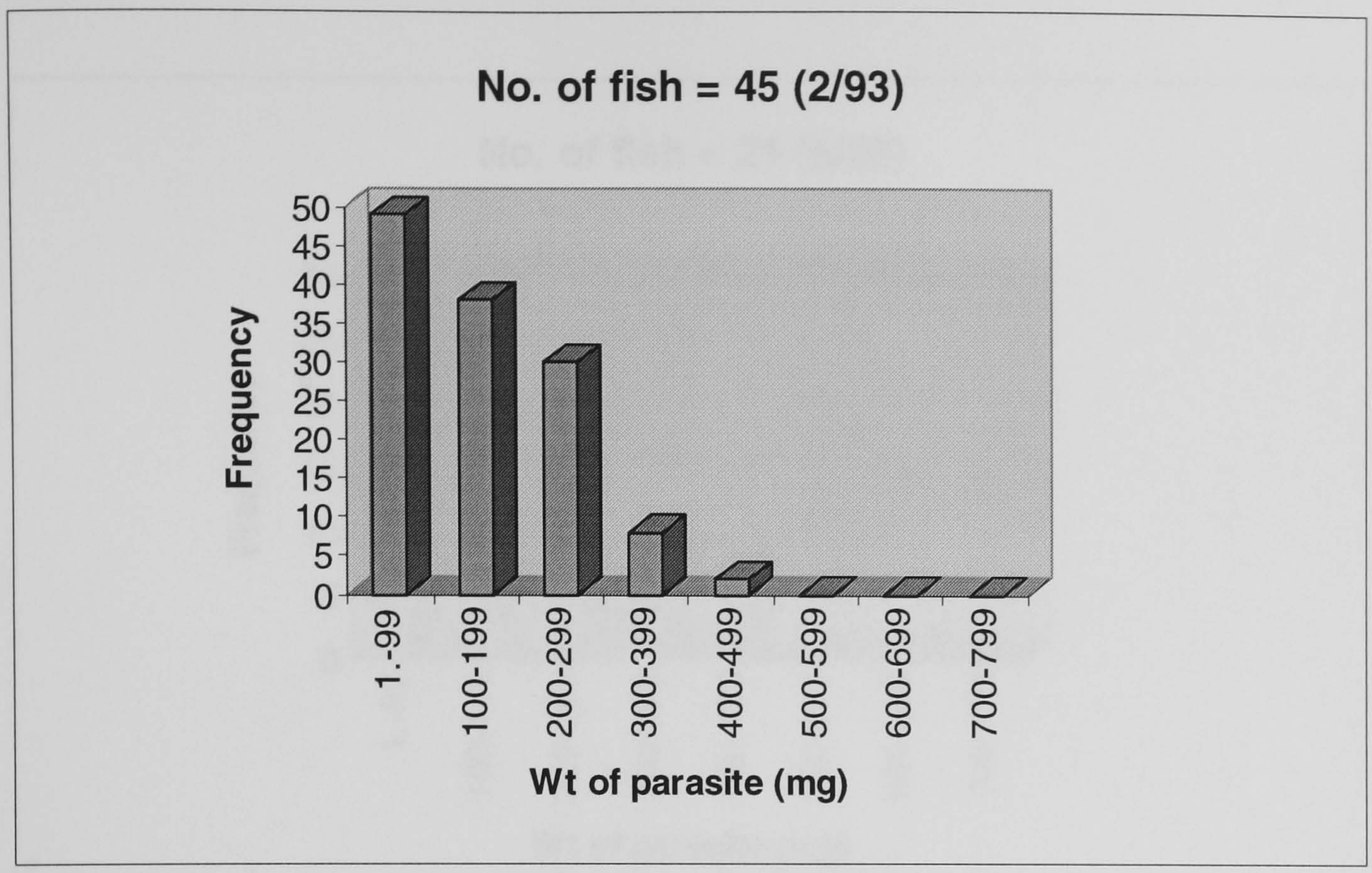


Figure 3.32: Weight frequency composition for February 1993 of *S. solidus* sample from site B.

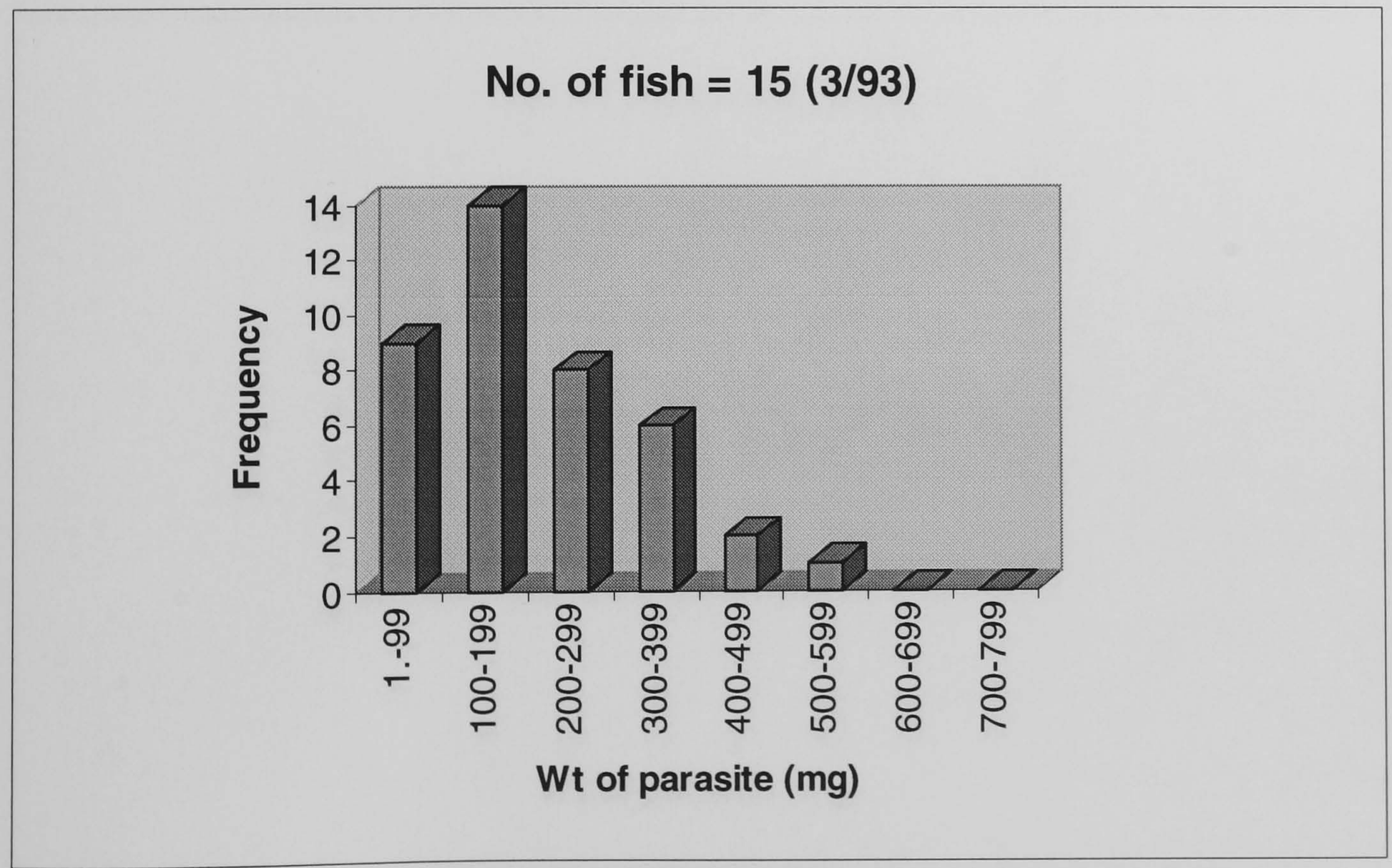


Figure 3.33: Weight frequency composition for March 1993 of *S. solidus* sample from site B.

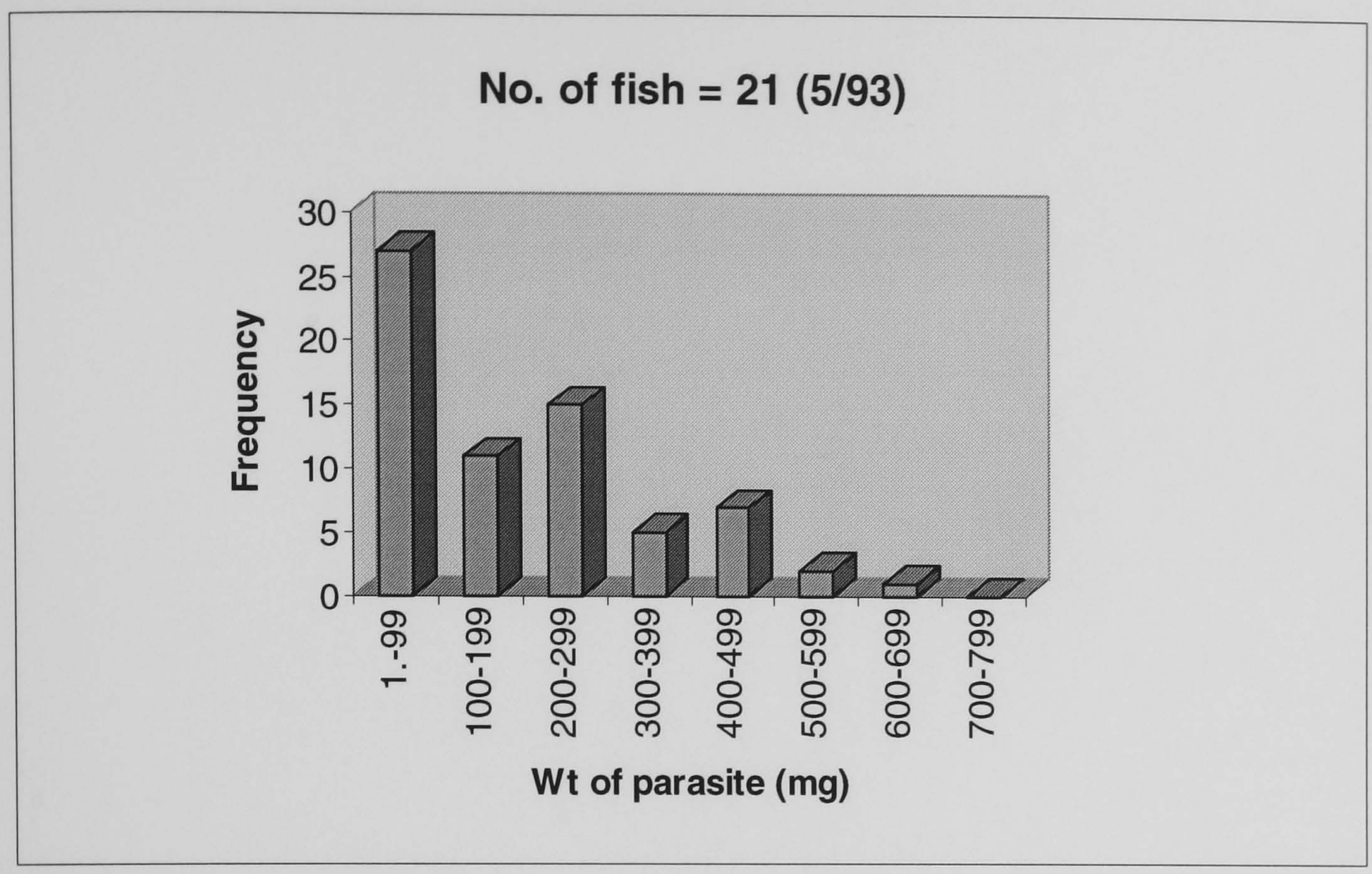


Figure 3.34: Weight frequency composition for May 1993 of *S. solidus* sample from site B.

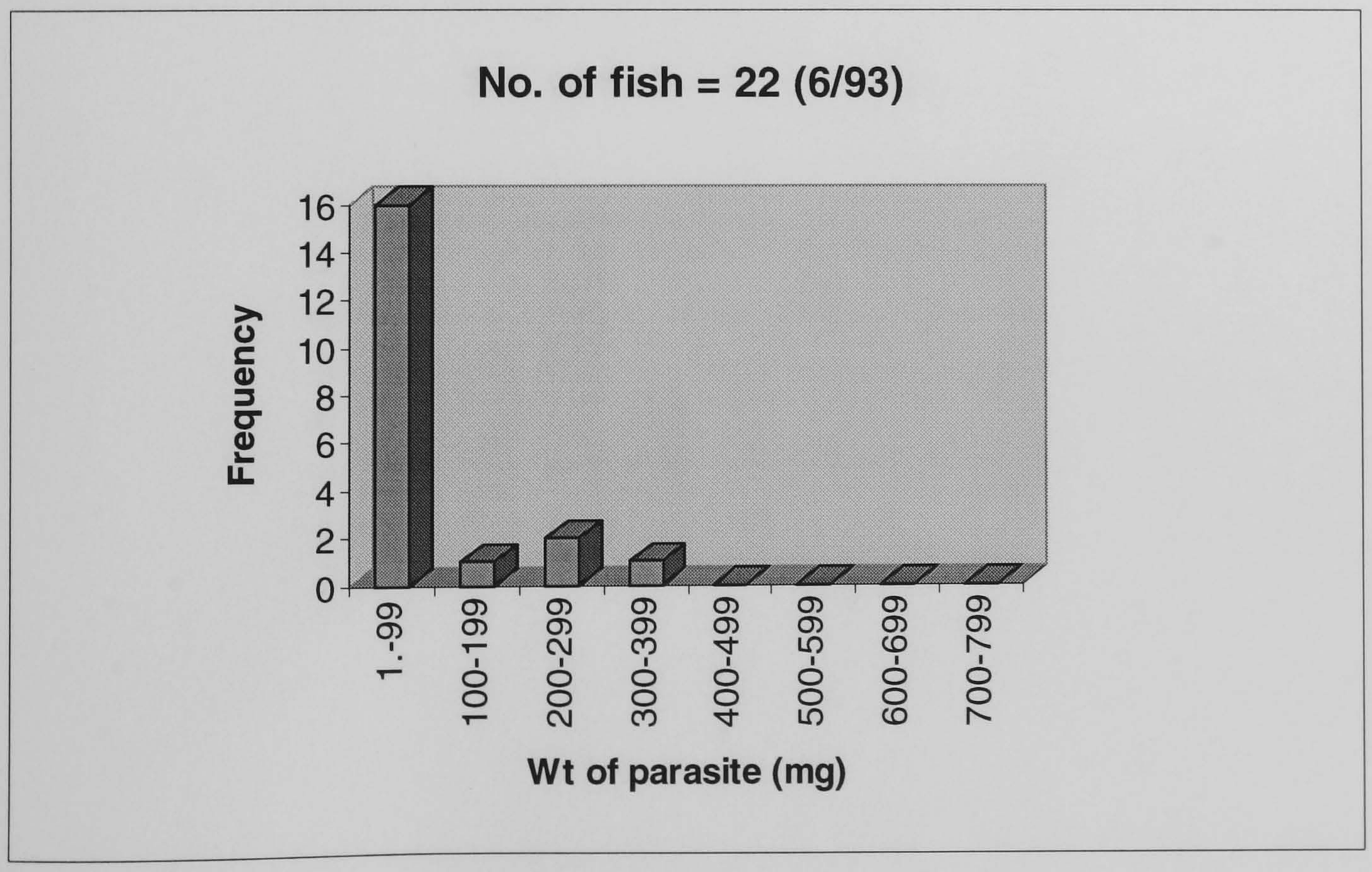


Figure 3.35: Weight frequency composition for June 1993 of *S. solidus* sample from site B.

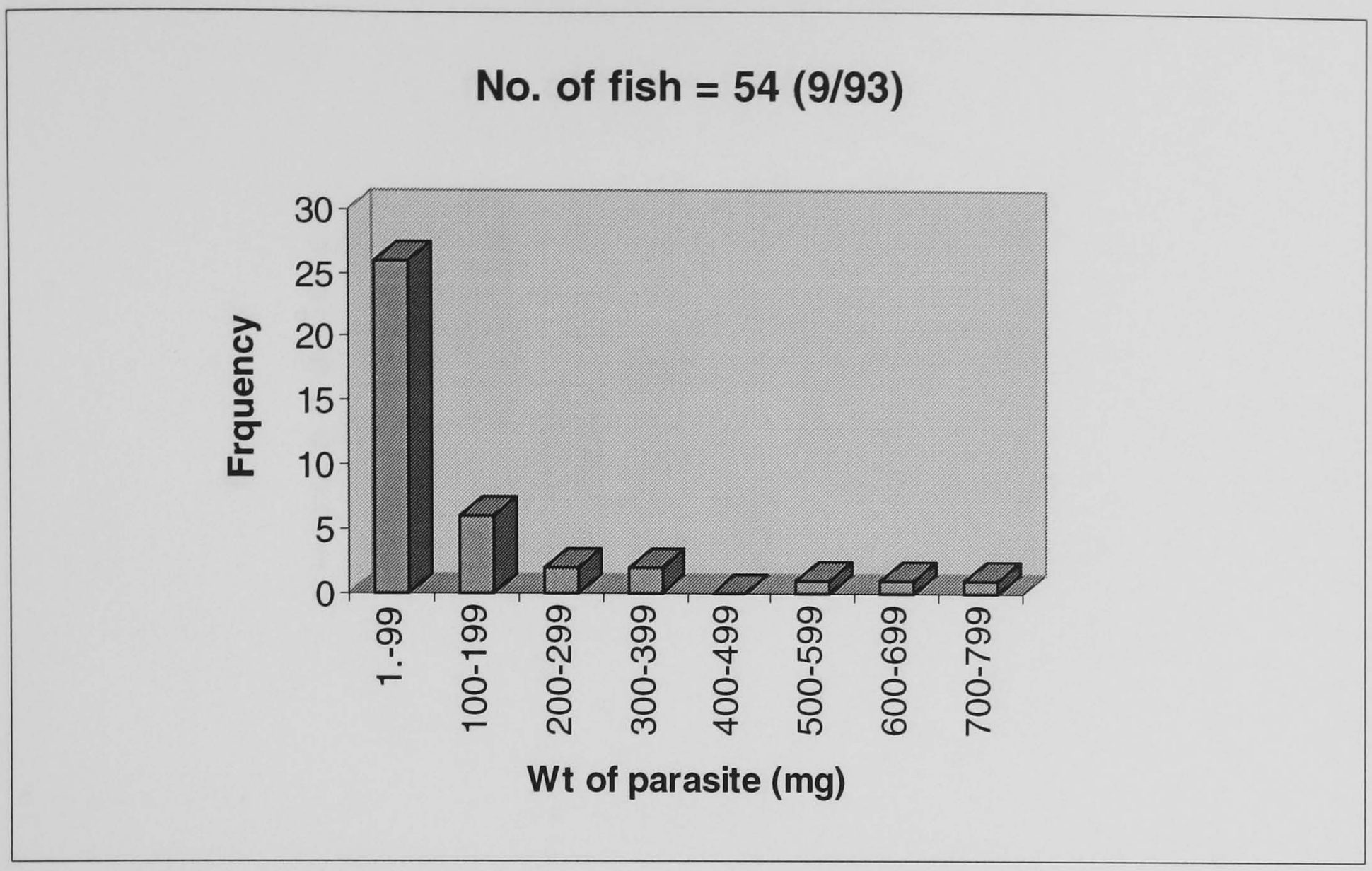


Figure 3.36: Weight frequency composition for September 1993 of *S. solidus* sample from site B.

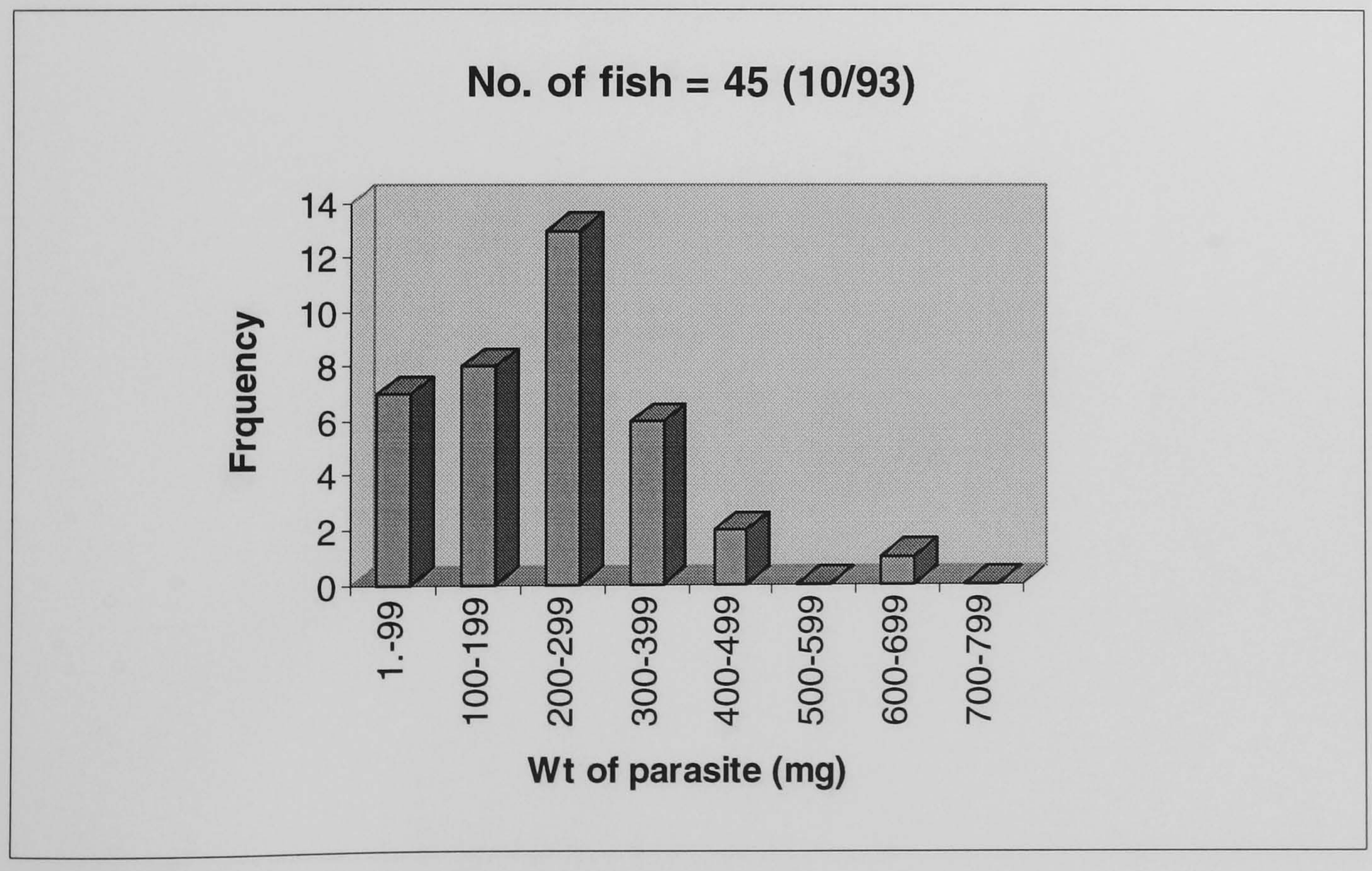


Figure 3.37: Weight frequency composition for October 1993 of *S. solidus* sample from site B.

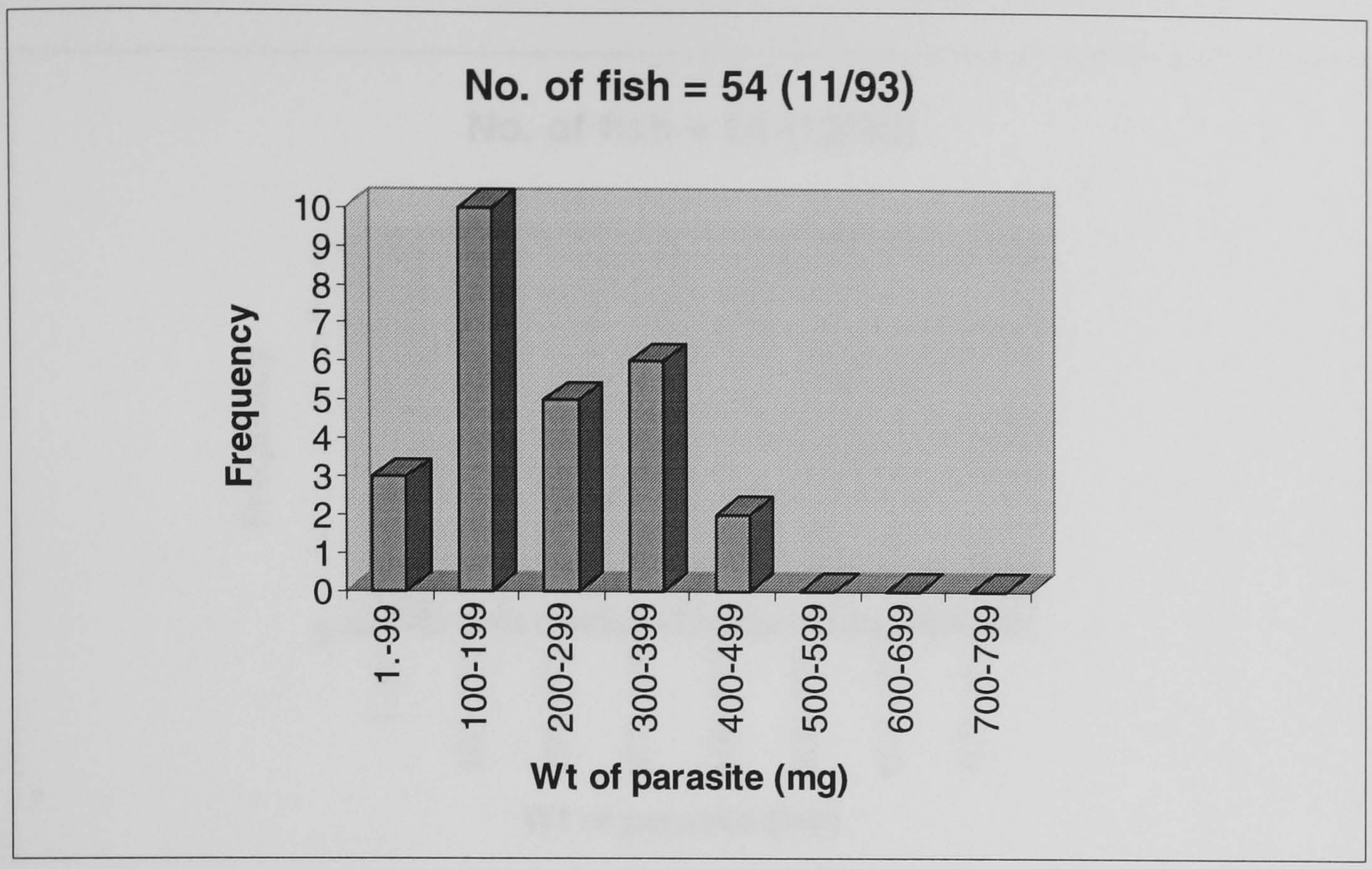


Figure 3.38: Weight frequency composition for November 1993 of *S. solidus* sample from site B.

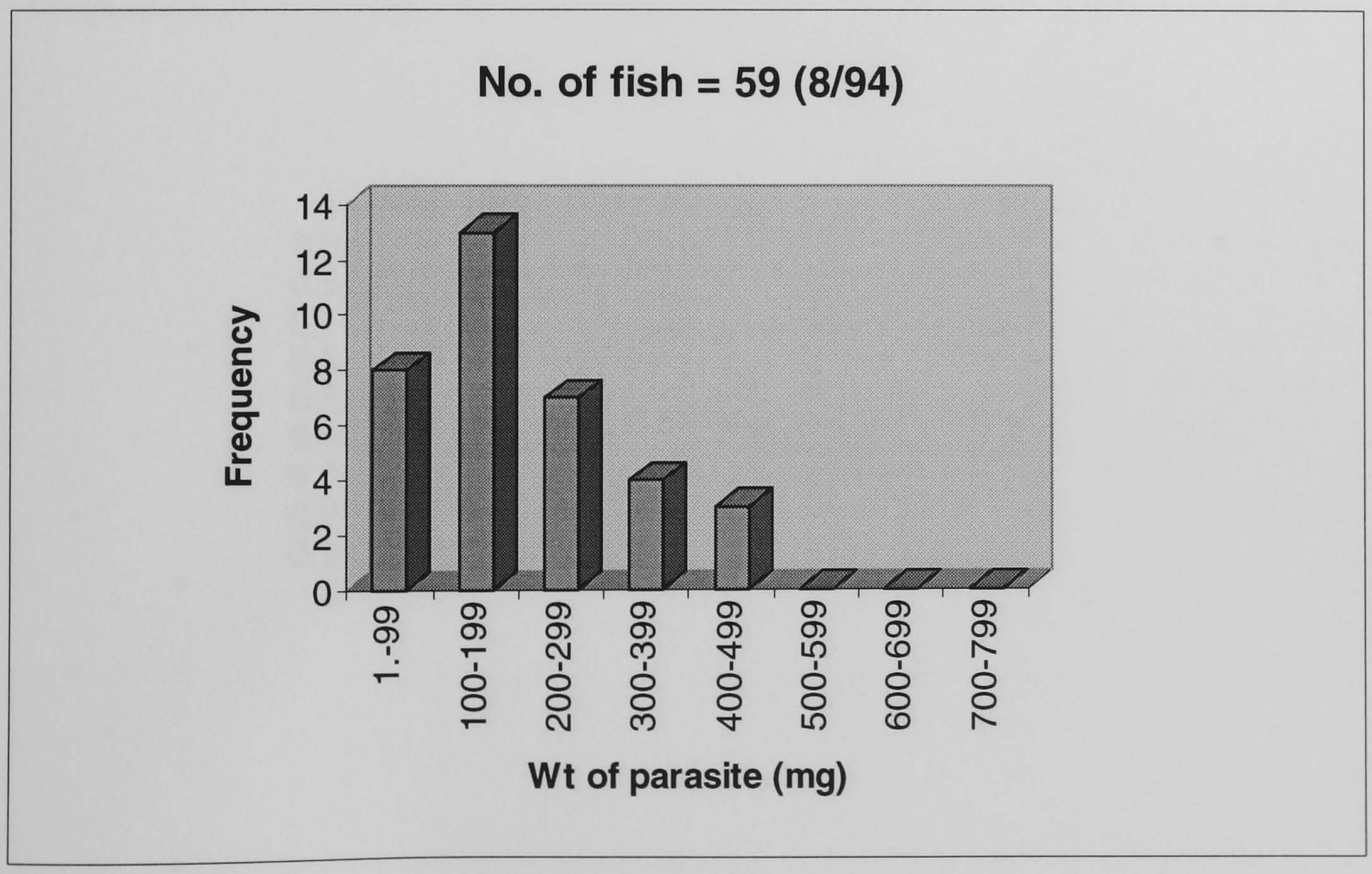


Figure 3.39: Weight frequency composition for August 1994 of *S. solidus* sample from site B.

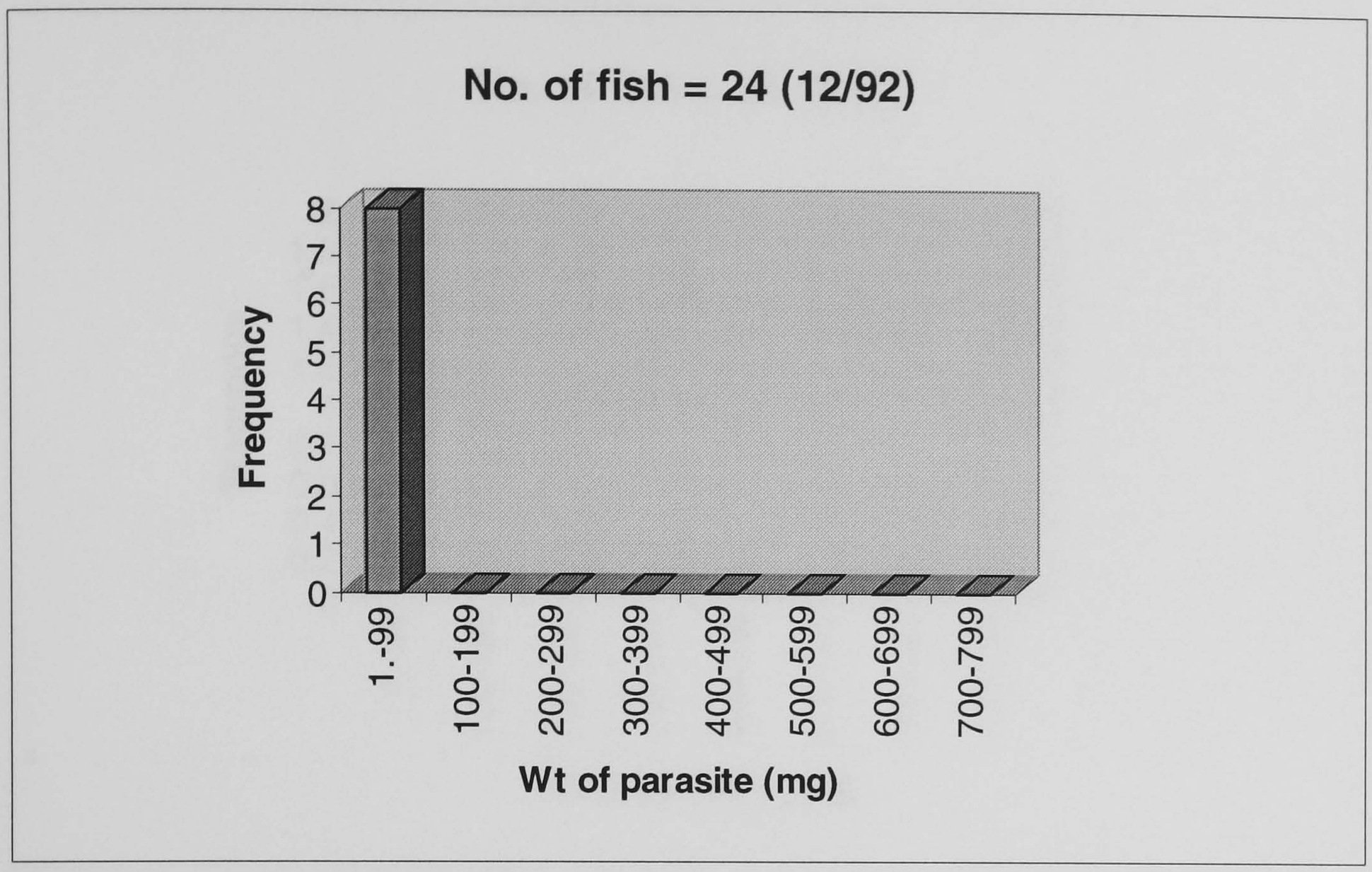


Figure 3.40: Weight frequency composition for December 1992 of *S. solidus* sample from site E.

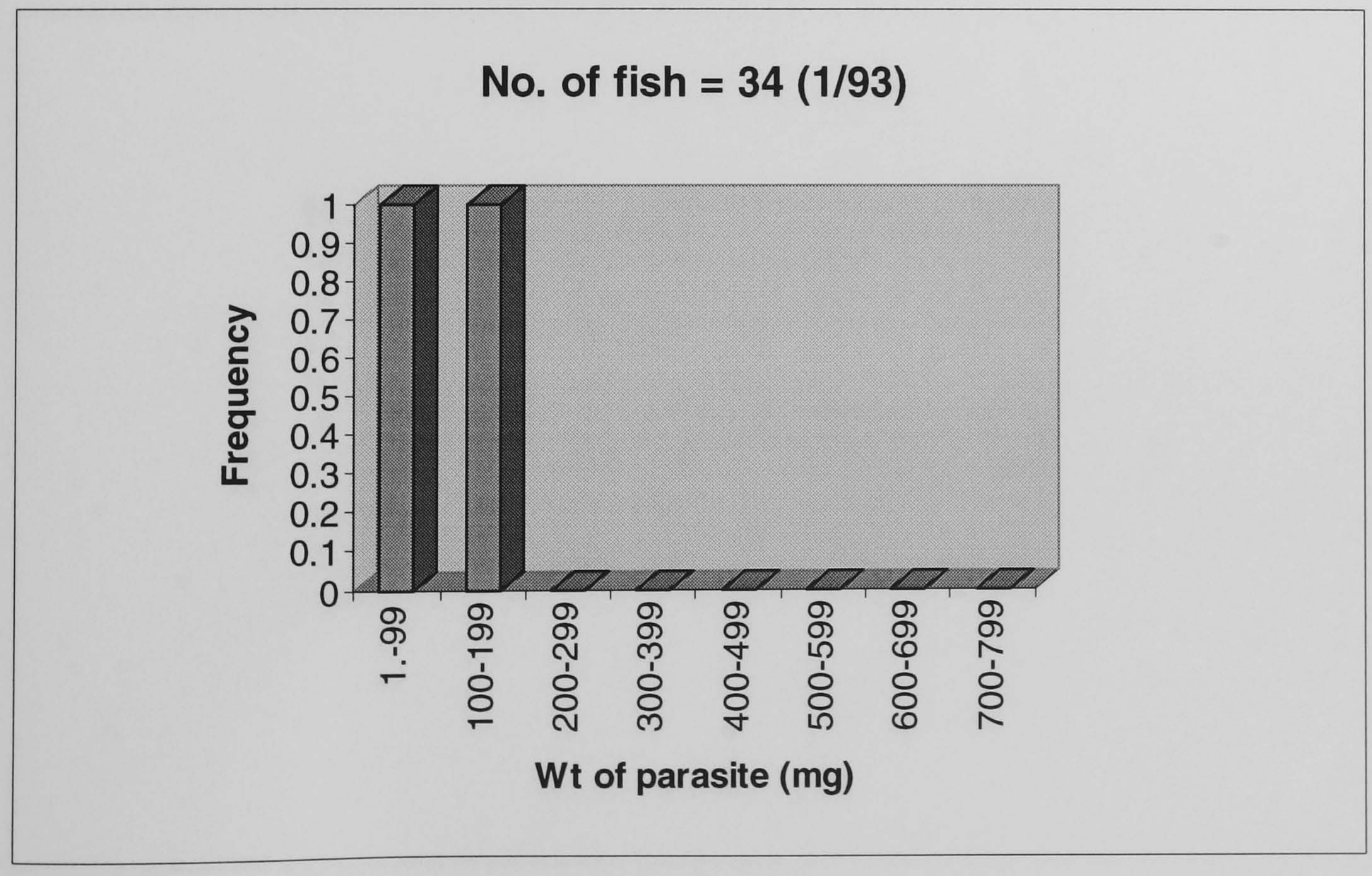


Figure 3.41: Weight frequency composition for January 1993 of *S. solidus* sample from site E.

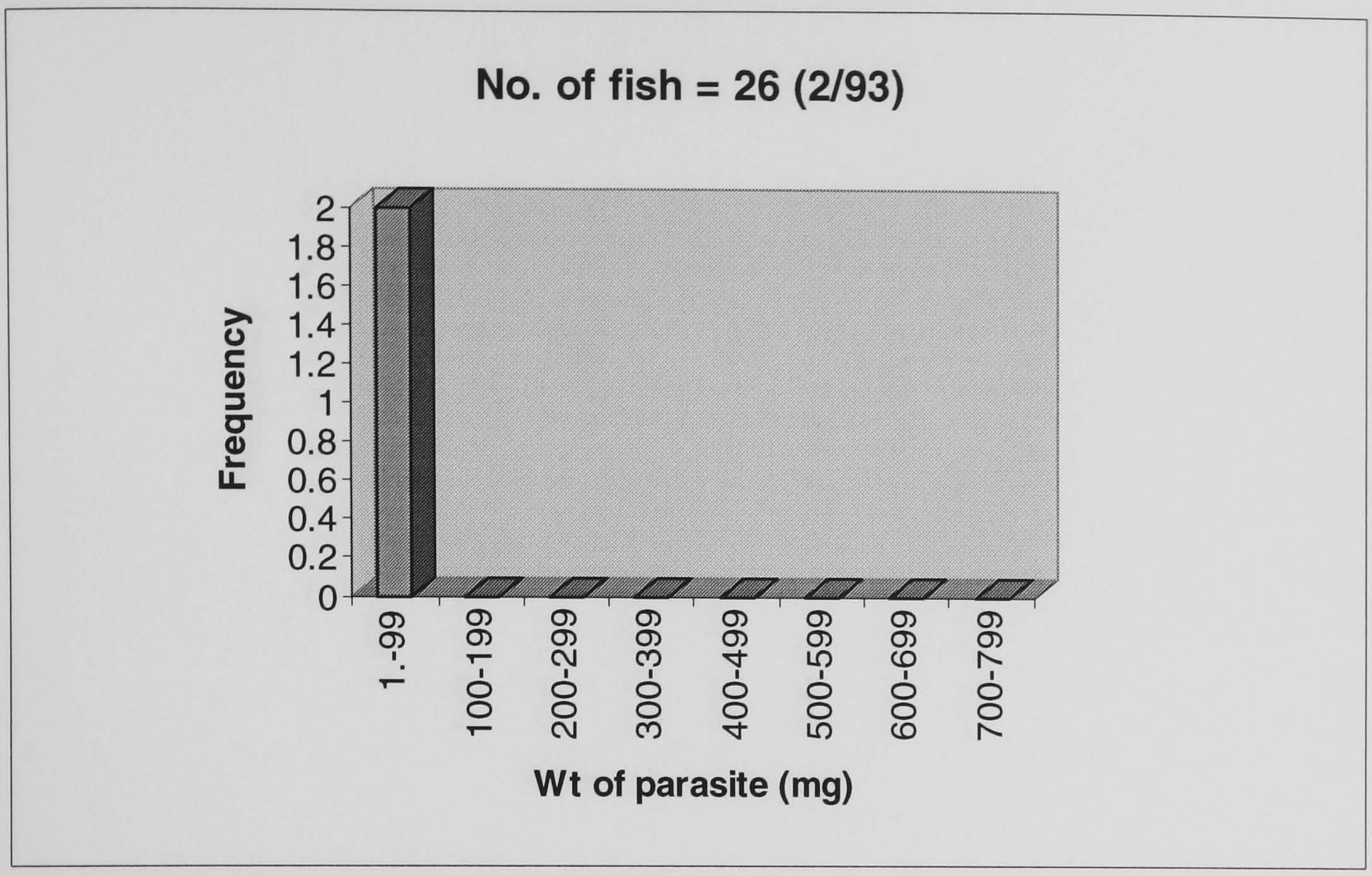


Figure 3.42: Weight frequency composition for February 1993 of *S. solidus* sample from site E.

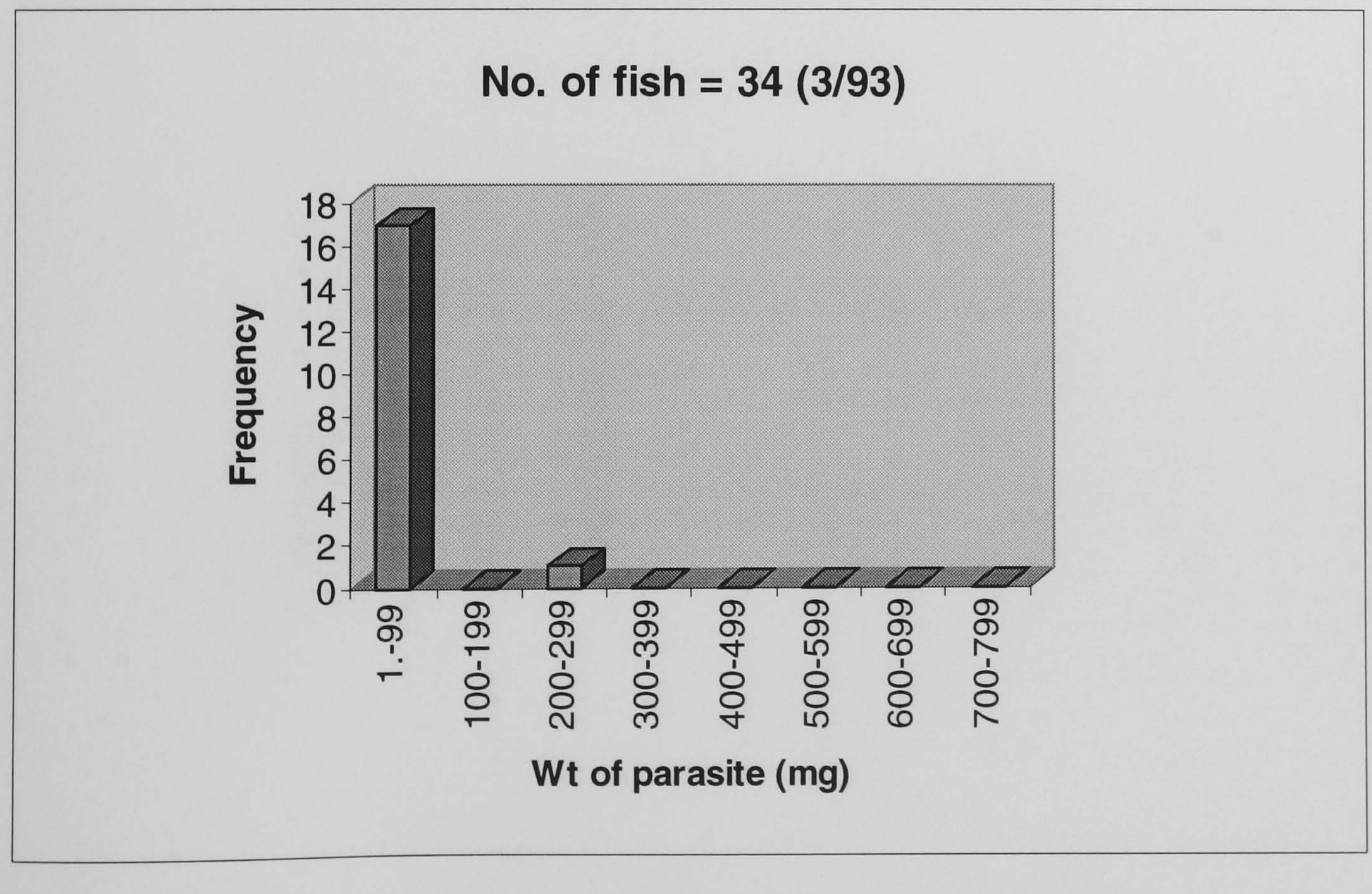


Figure 3.43: Weight frequency composition for March 1993 of *S. solidus* sample from site E.

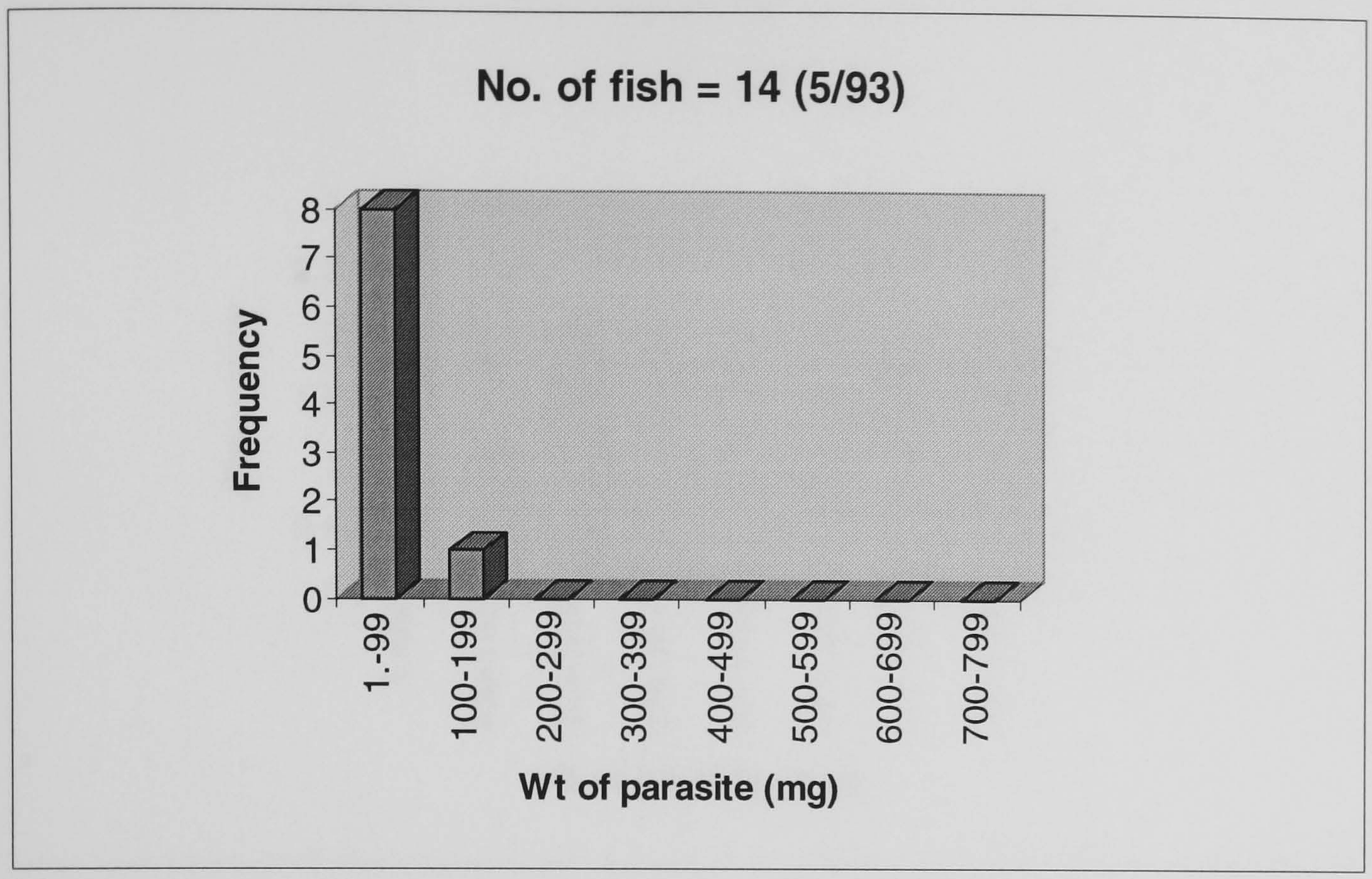


Figure 3.44: Weight frequency composition for May 1993 of *S. solidus* sample from site E.

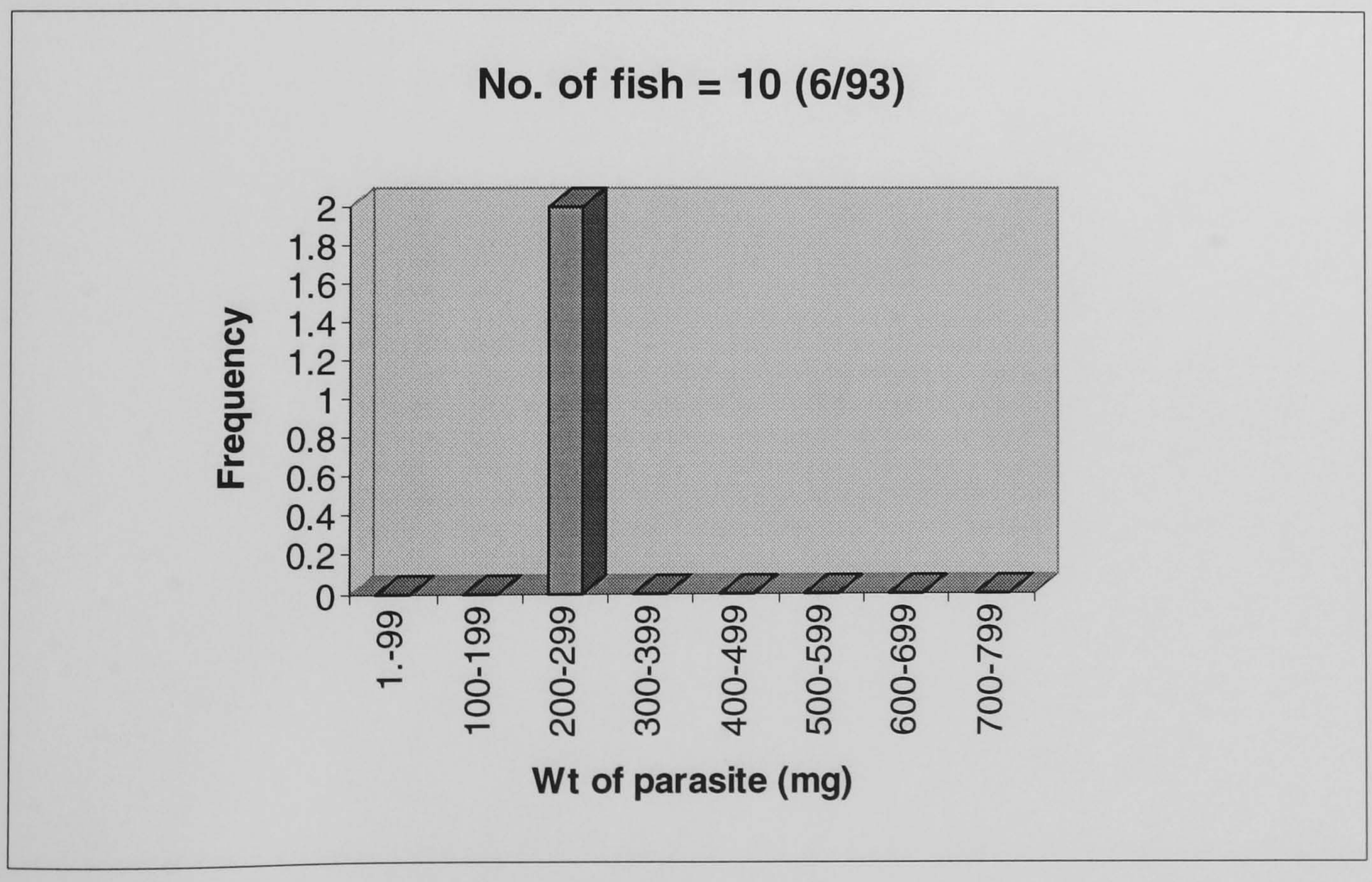


Figure 3.45: Weight frequency composition for June 1993 of *S. solidus* sample from site E.

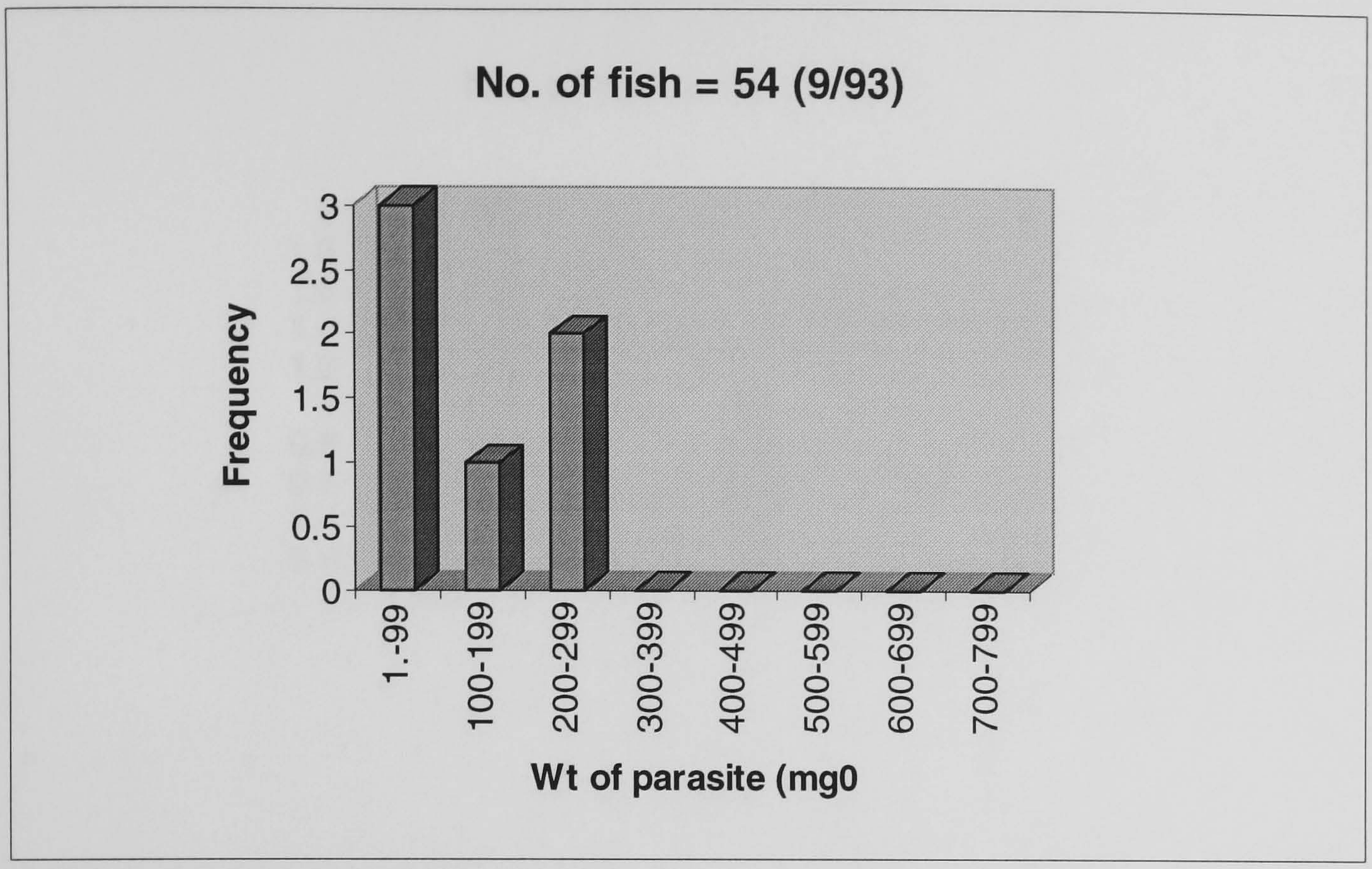


Figure 3.46: Weight frequency composition for September 1993 of *S. solidus* sample from site E.

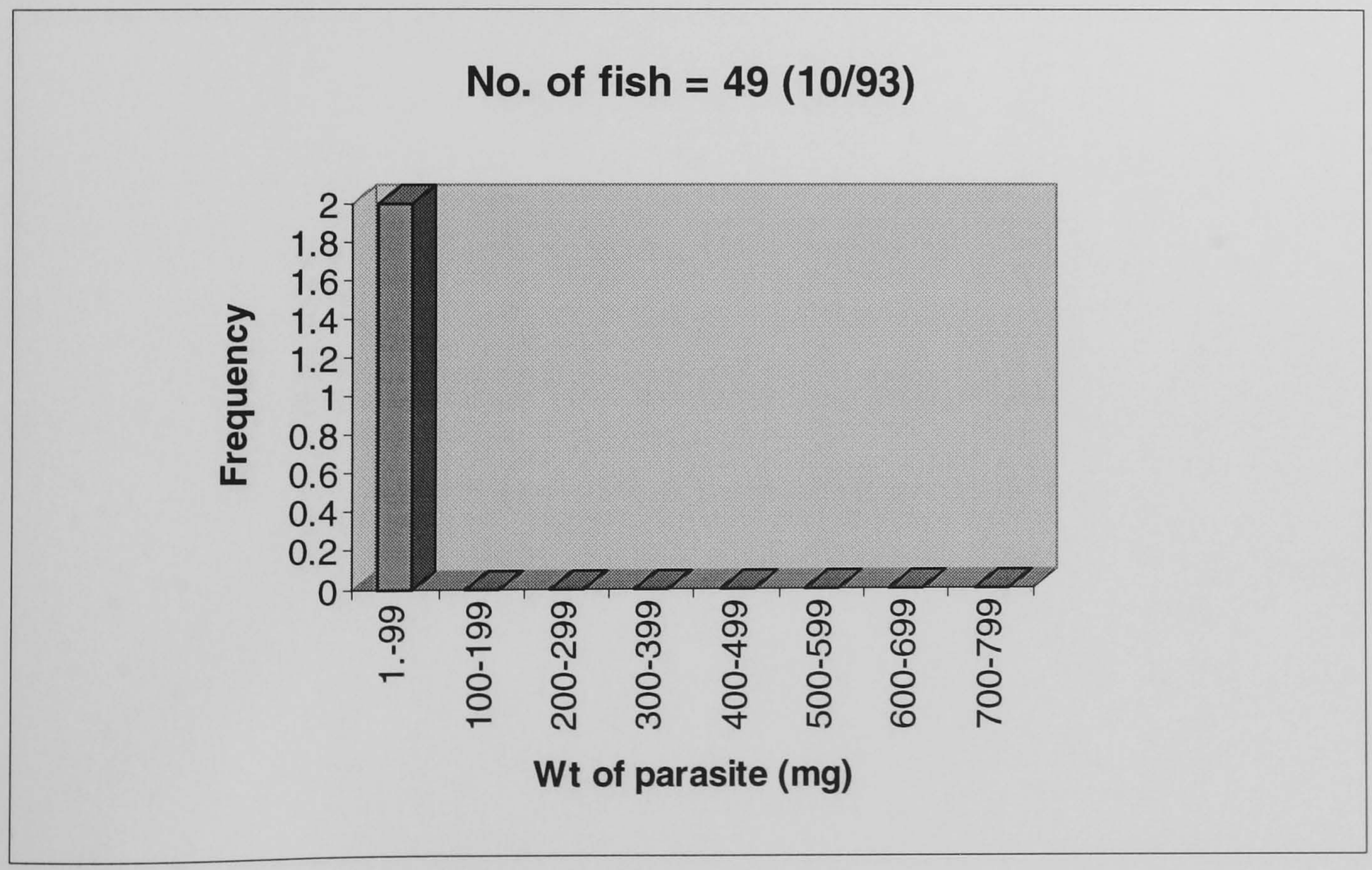


Figure 3.47: Weight frequency composition for October 1993 of *S. solidus* sample from site E.

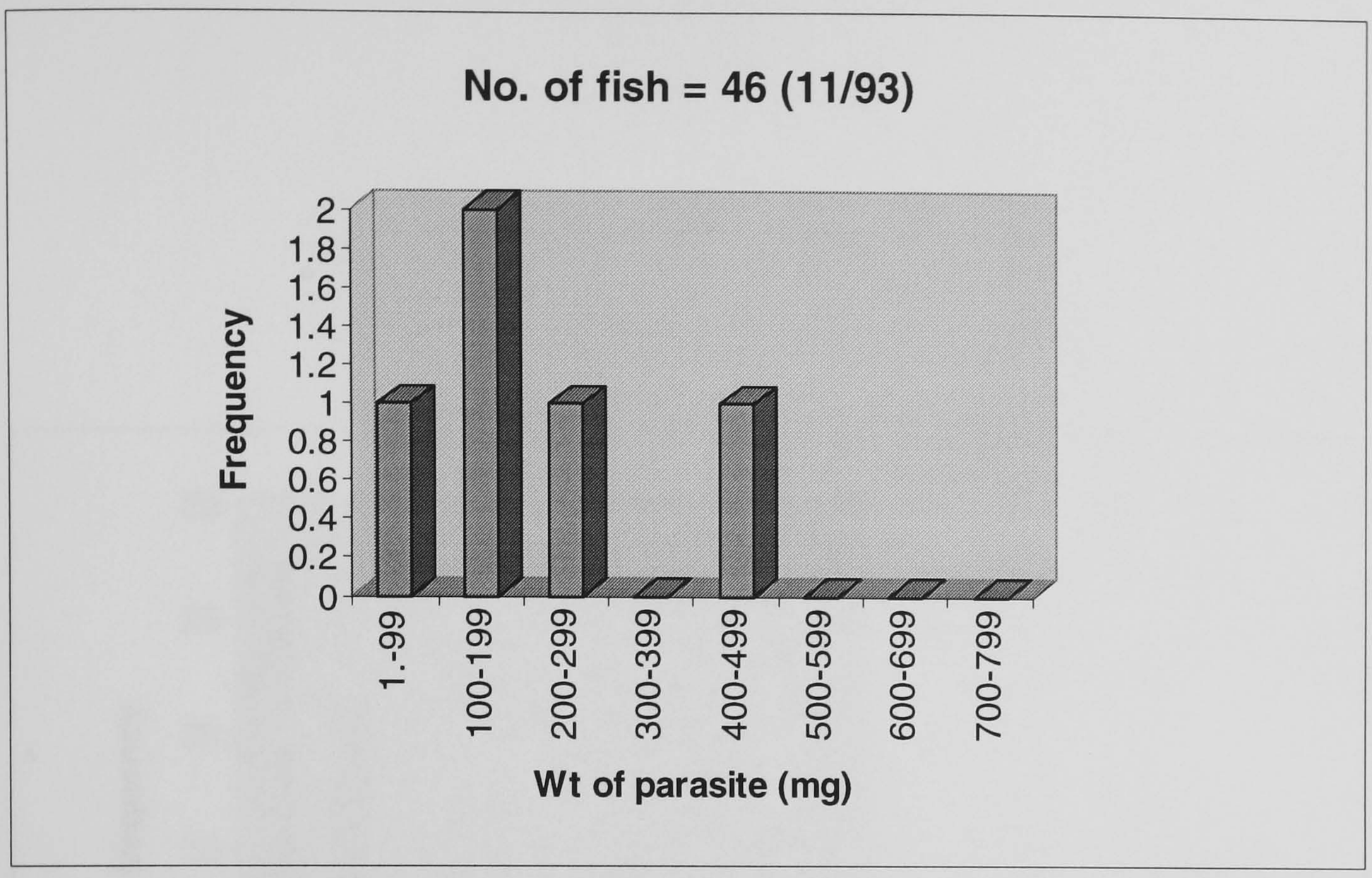


Figure 3.48: Weight frequency composition for November 1993 of *S. solidus* sample from site E.

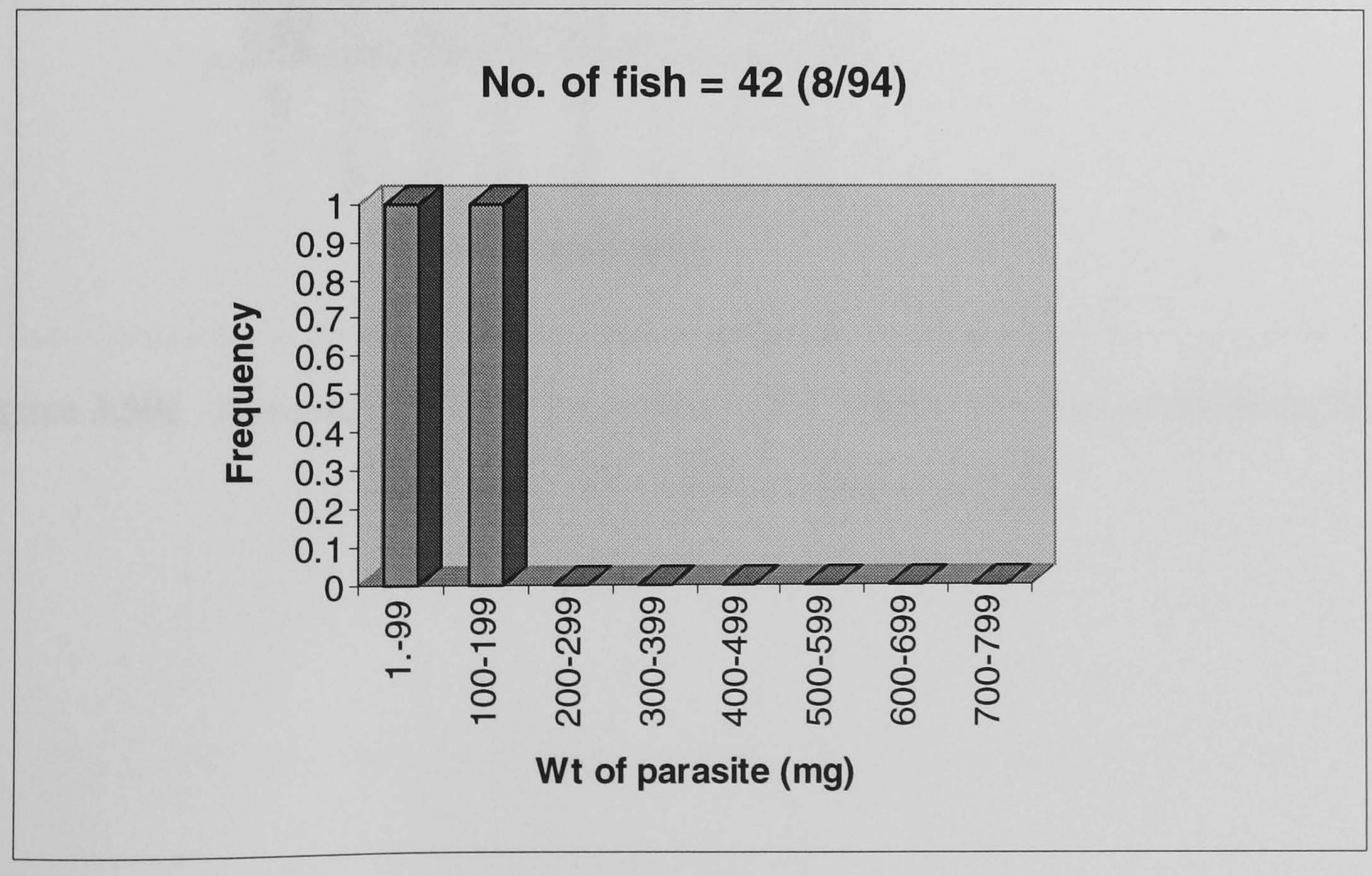


Figure 3.49: Weight frequency composition for August 1994 of *S. solidus* sample from site E.

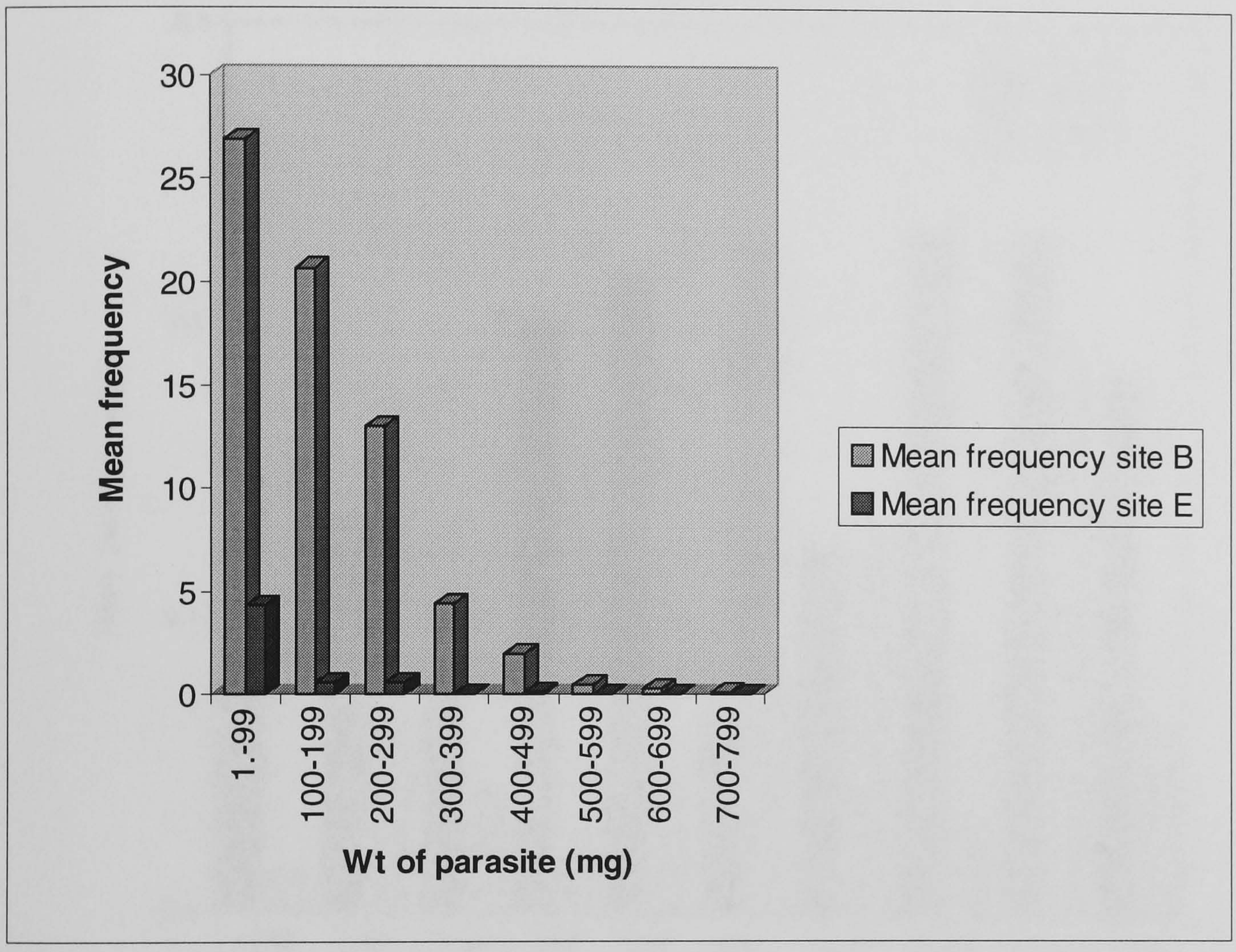


Figure 3.50: Mean weight class frequencies of *S. solidus* infection at site B and E.

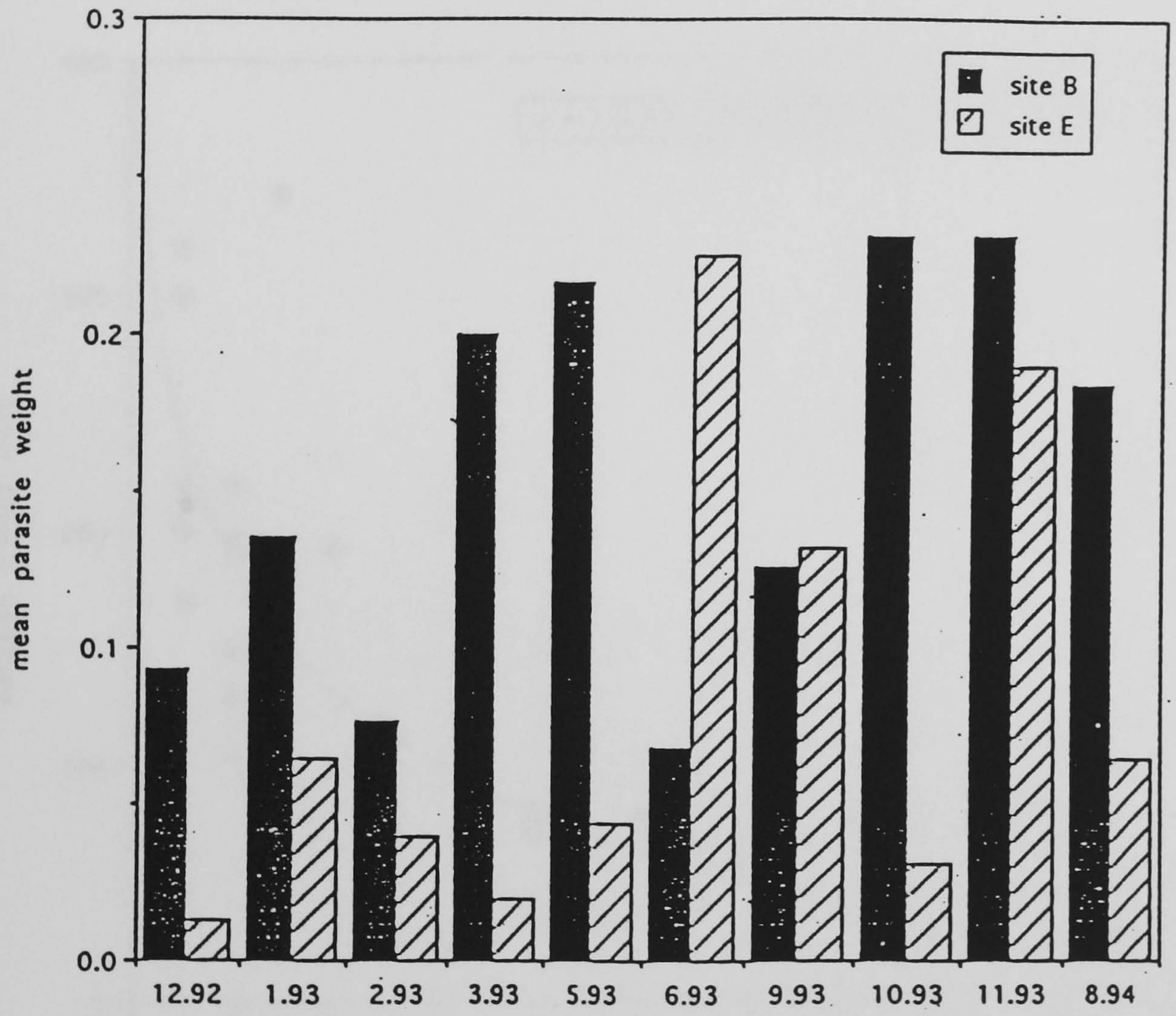


Figure 3.51: Mean total parasite weight in sticklebacks from sites B and E.

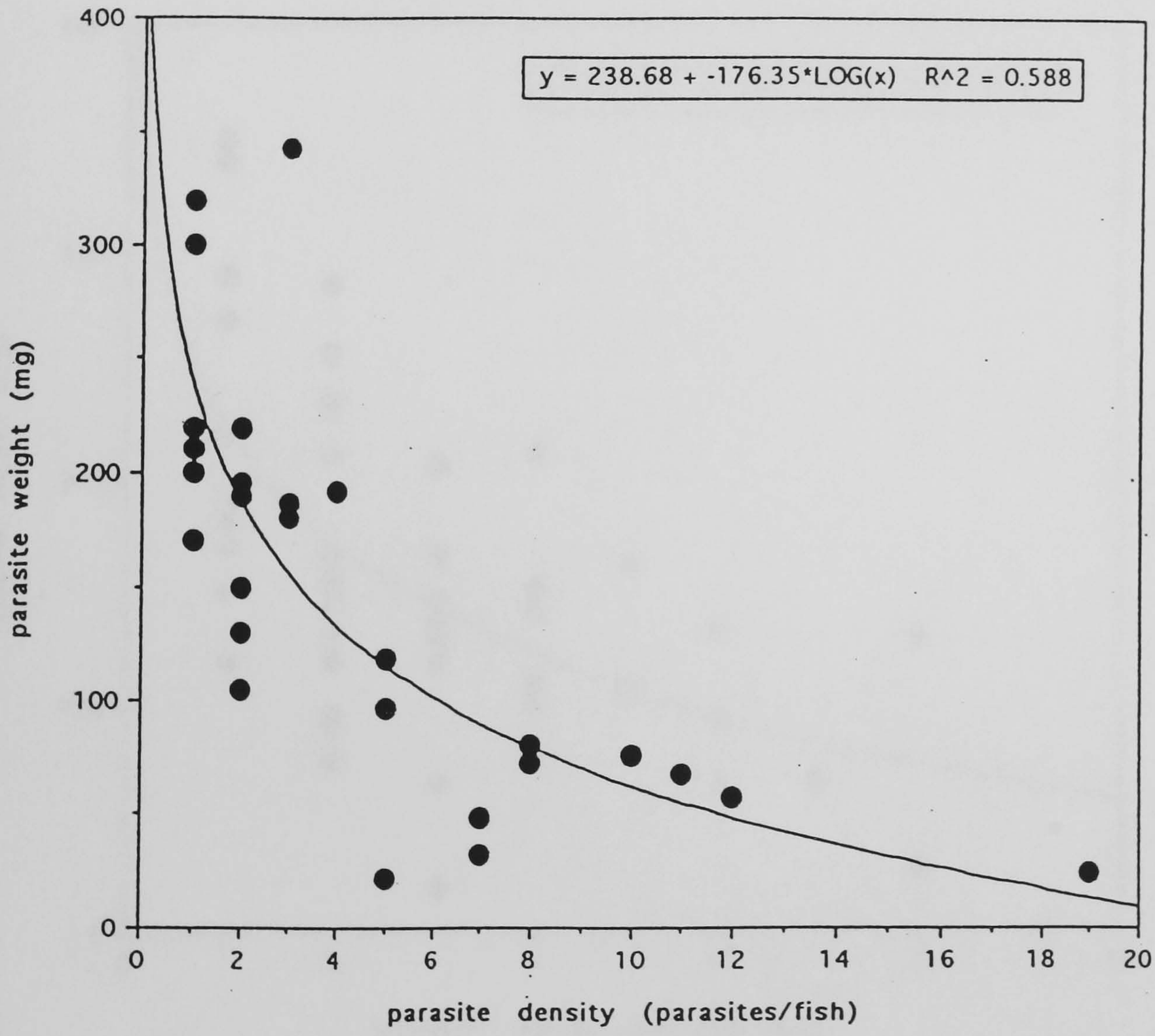


Figure 3.52: Relationship between parasite density (parasite/fish) and mean parasite weight *S. solidus* in the December 1992 sample from site B.

of

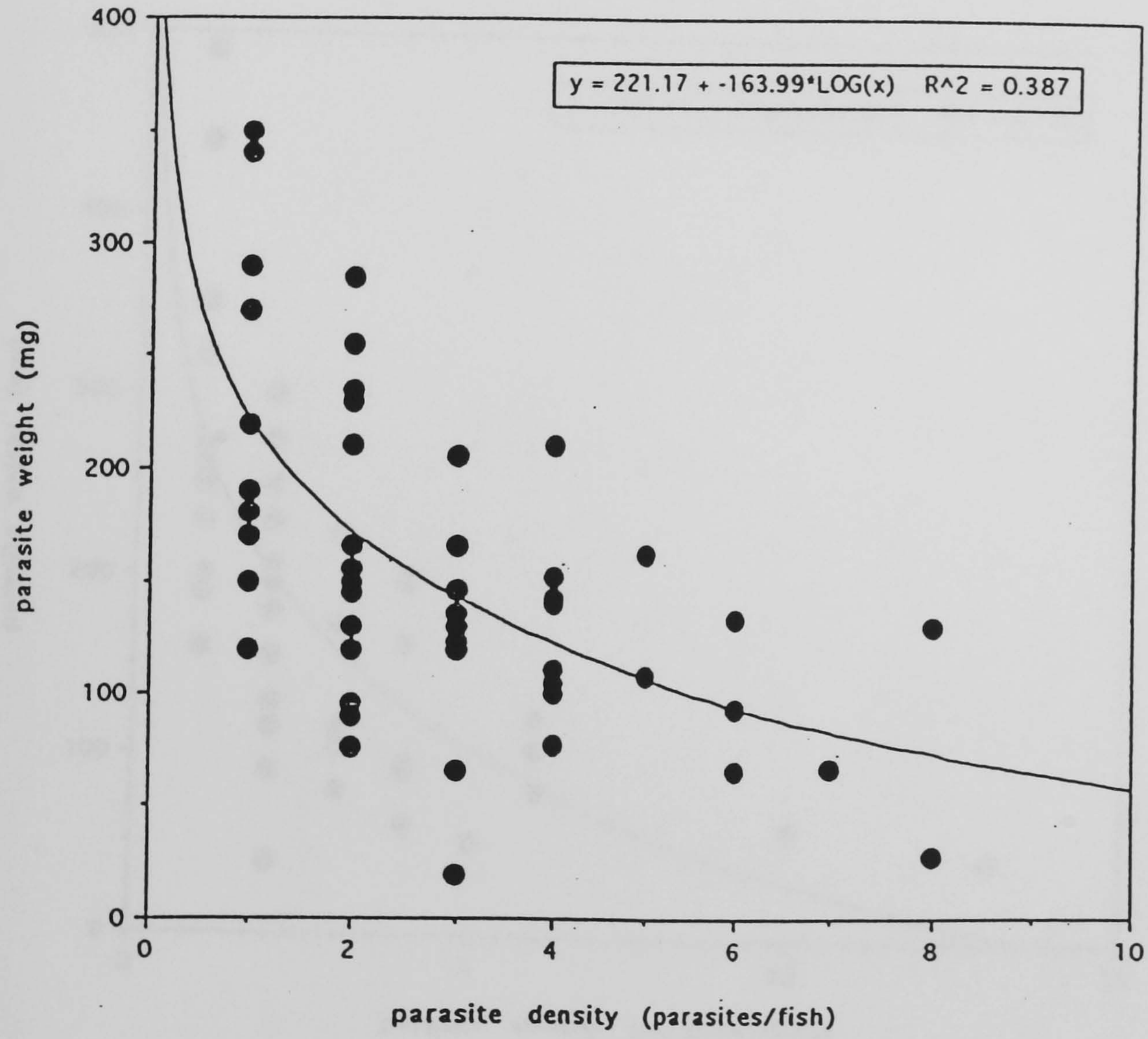


Figure 3.53: Relationship between parasite density (parasite/fish) and mean parasite weight of *S. solidus* in the January 1993 sample from site B.

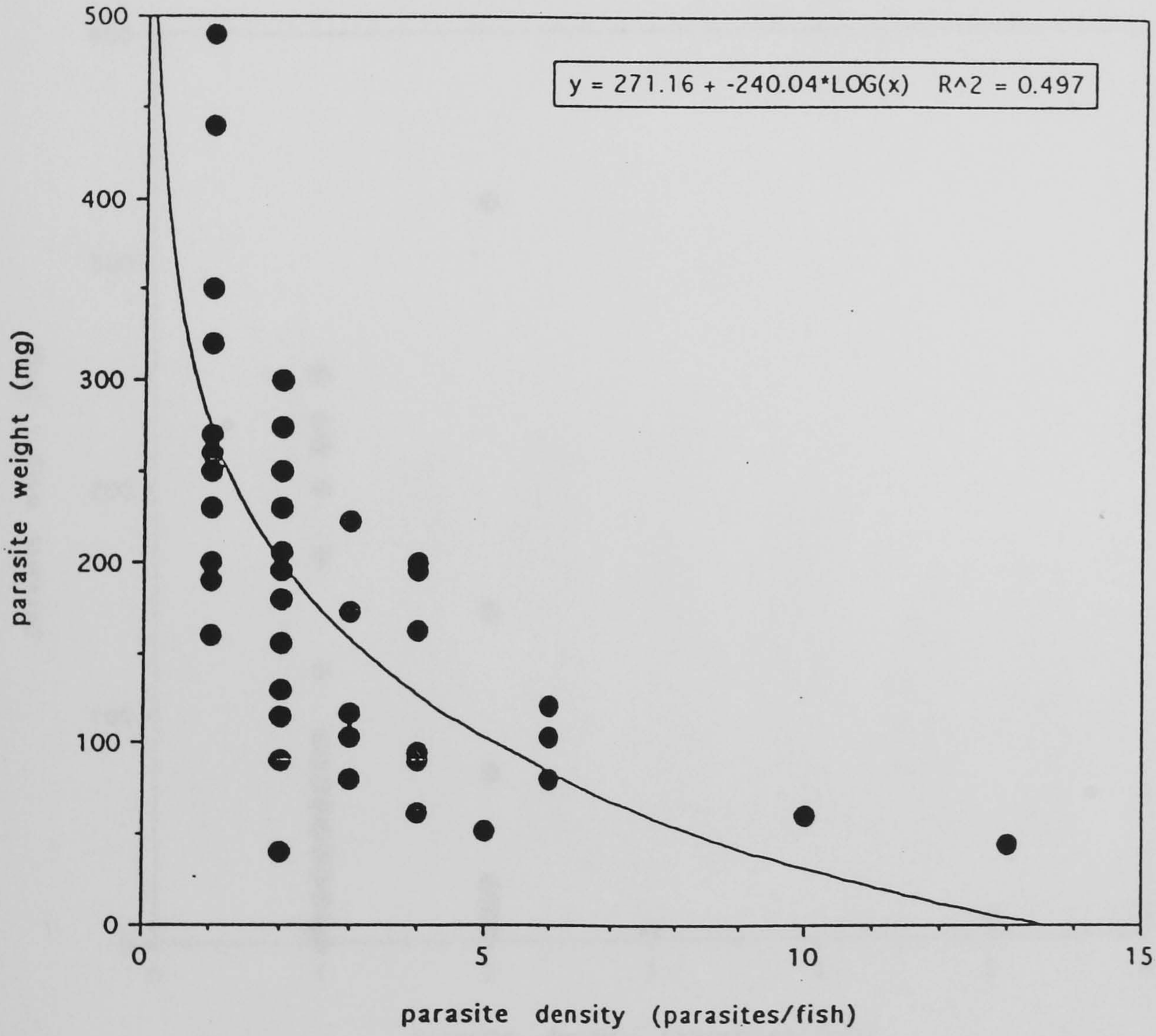


Figure 3.54: Relationship between parasite density (parasite/fish) and mean parasite weight of *S. solidus* in the February 1993 sample from site B.

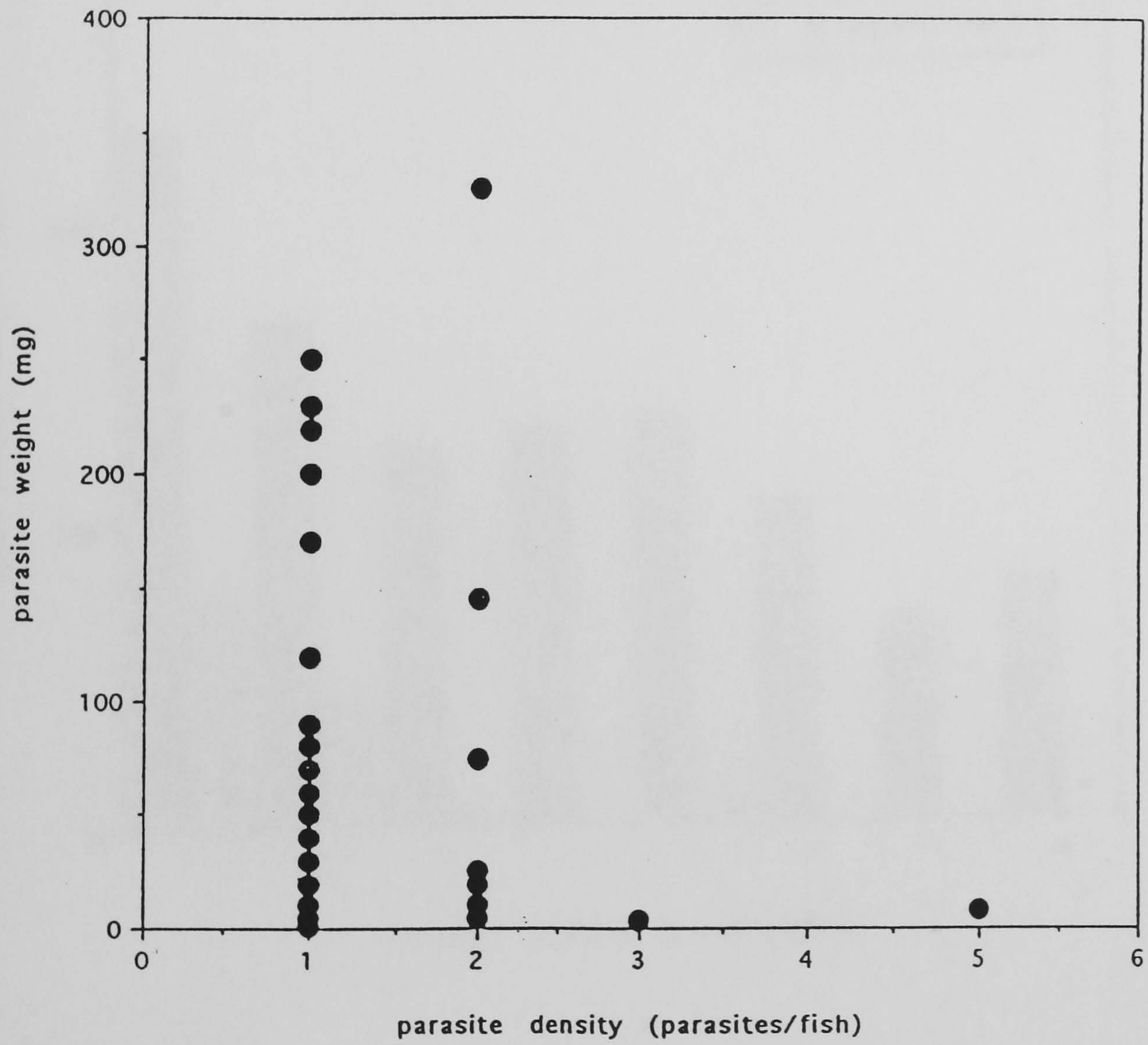


Figure 3.55: Relationship between parasite density (parasite/fish) and mean parasite weight of *S. solidus* in all samples from December 1992 till August 1994 at site E.

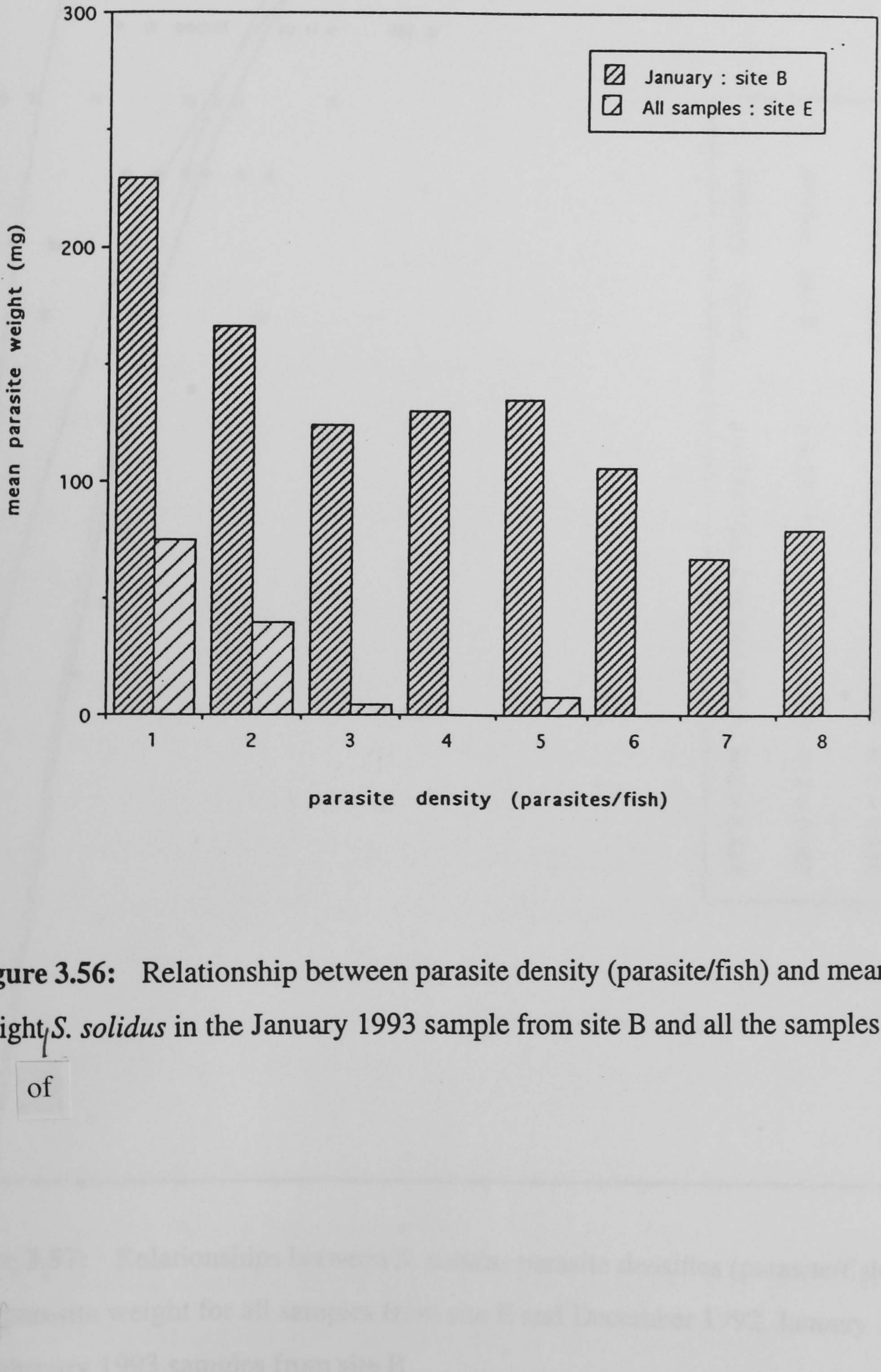


Figure 3.56: Relationship between parasite density (parasite/fish) and mean parasite weight of *S. solidus* in the January 1993 sample from site B and all the samples from site E.

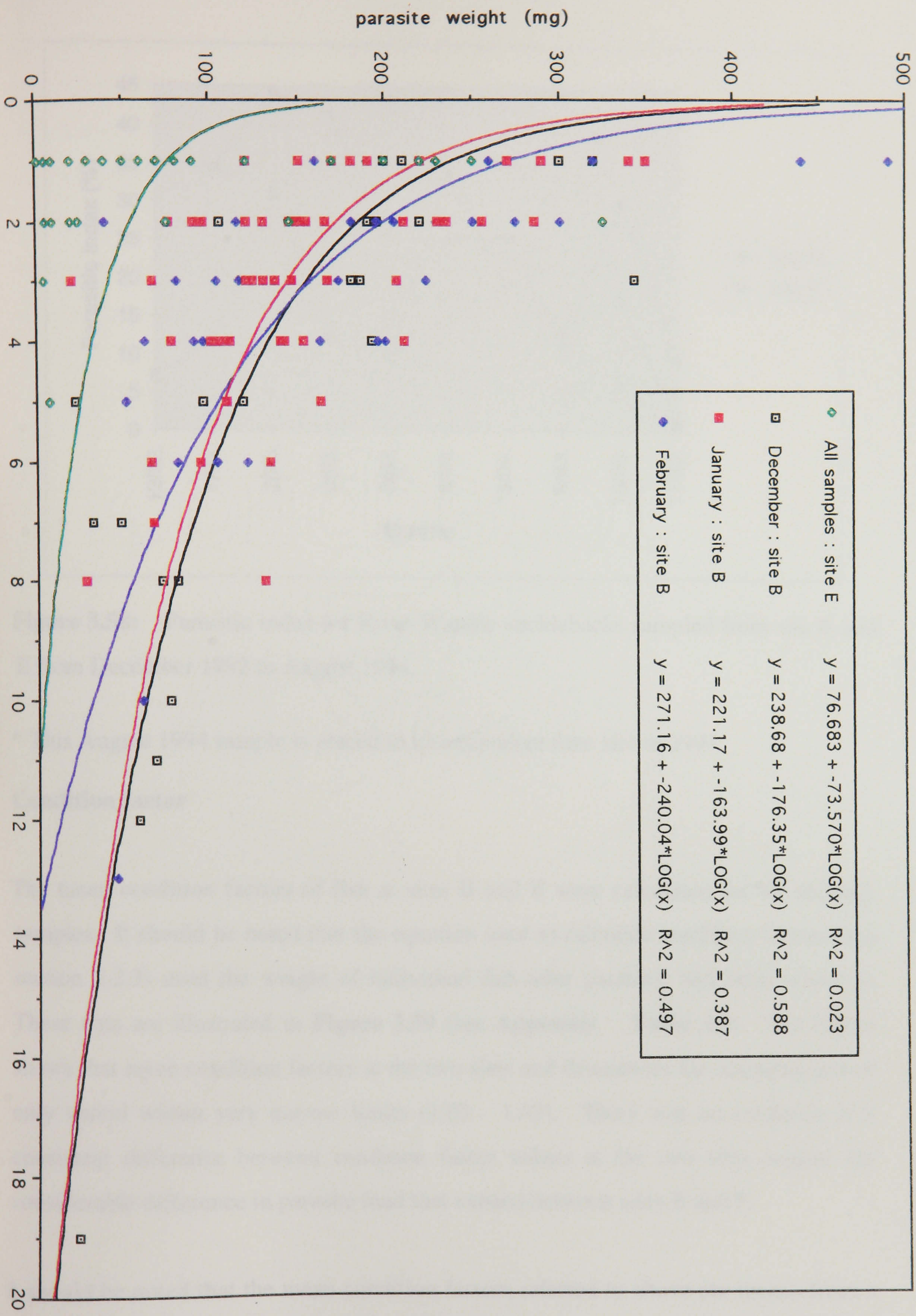


Figure 3.57: Relationships between *S. solidus* parasite densities (parasite/fish) and mean parasite weight for all samples from site E and December 1992, January 1993 and February 1993 samples from site B.

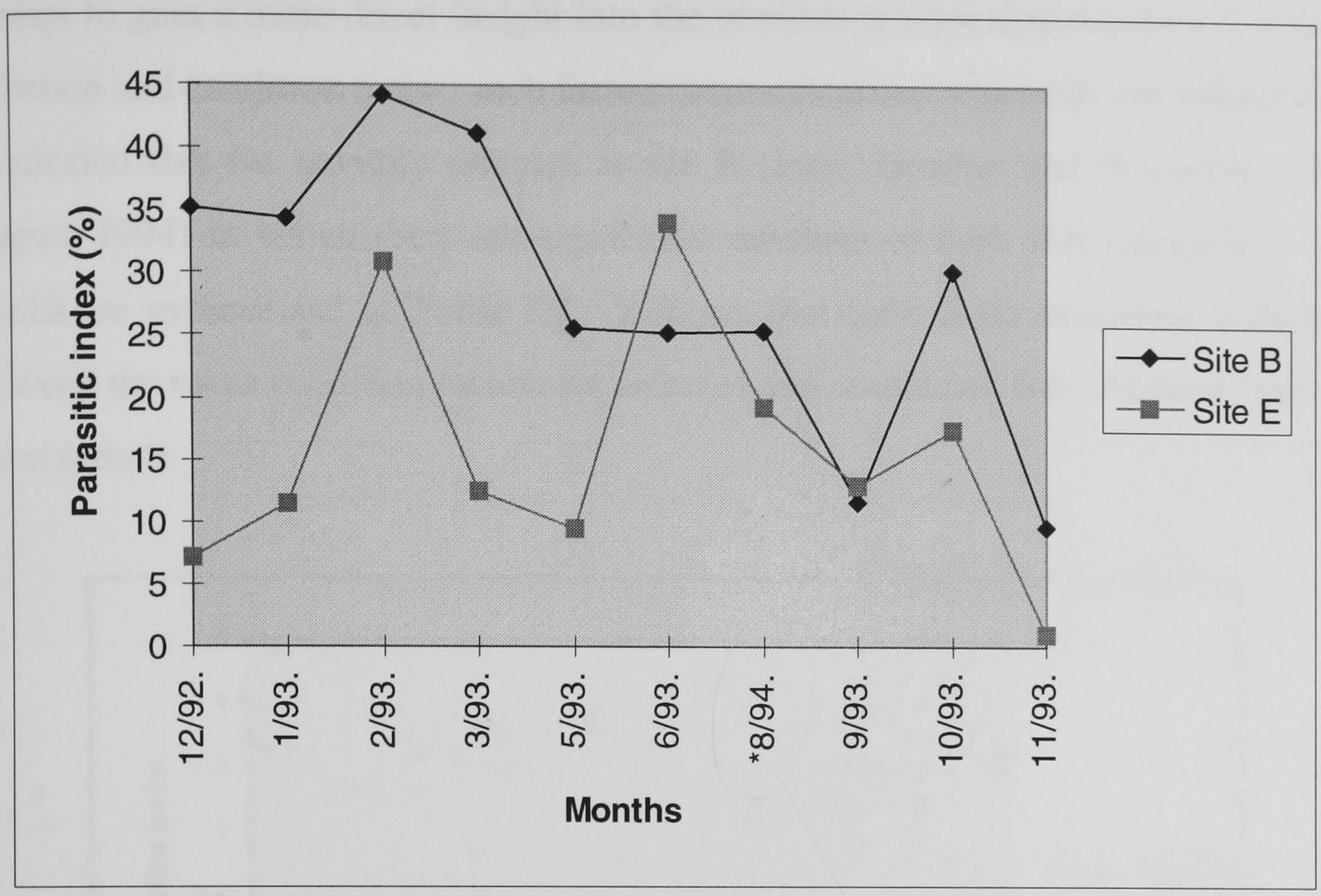


Figure 3.58: Parasitic index for River Wandle sticklebacks sampled from site B and E from December 1992 to August 1994.

* This August 1994 sample is placed in its equivalent time slot in 1993.

Condition factor

The mean condition factors of fish at sites B and E were calculated for all monthly samples. It should be noted that the equation used to calculate condition factors (see section 3.2.3) used the weight of individual fish after parasites had been removed. These data are illustrated in **Figure 3.59** (see **Appendix Table A3**). The Figure shows that mean condition factors at the two sites and throughout the sampling period only varied within very narrow limits (1.03 - 1.43). There was no evidence of a consistent difference between condition factor values at the two sites despite the considerable difference in parasite load that existed between sites B and E.

It should be noted that the mean condition factors referred to above are means from a mixture of parasitised and unparasitised fish. Conclusions as to whether parasitisation might be affecting condition factor can only be drawn indirectly on the basis of the differential infection prevalences and intensities in the different samples. In an

attempt to gain a more direct insight into the possible relationship between *S. solidus* infection and condition factor, such factors were calculated separately for infected and uninfected fish for monthly samples at site B (June, October and November 1993, August 1994) in which there are significant numbers of both fish categories. The results are summarised in **Table 3.9.** It shows that there is no consistent difference between the mean condition factors for infected and uninfected fish obtained from the same sample.

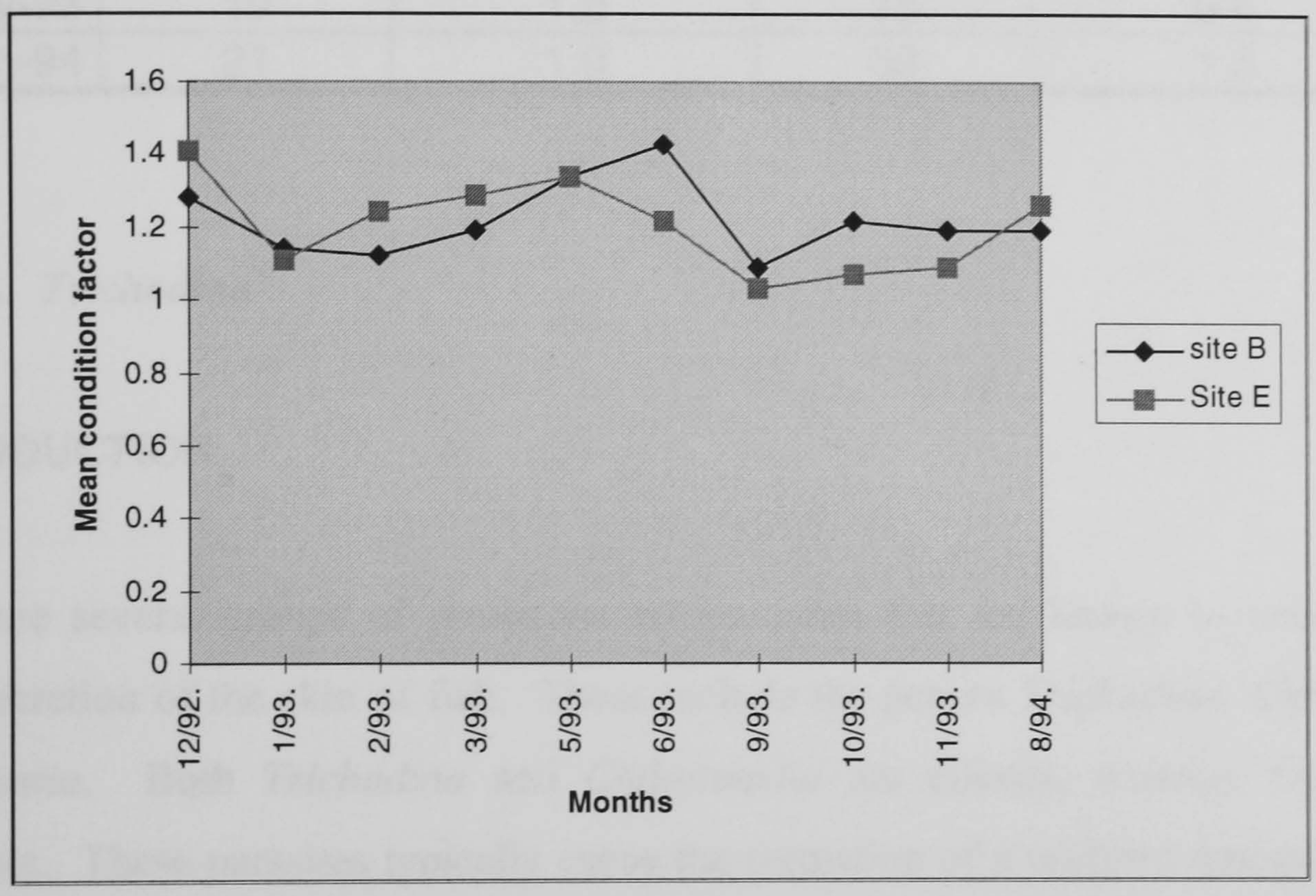


Figure 3.59: Mean condition factor for River Wandle sticklebacks sampled from site B and E during December 1992 to August 1994.

Table 3.4: A comparison of mean condition factor of infected and uninfected fish in four months sample (June/92, Oct/93, Nov/93 and Aug/94) from site B.

	Infected		Uninfected	
Months	**Total fish no.**	**Mean condition factor**	**Total fish no.**	**Mean condition factor**
Jun-93	5	1.4	17	1.4
Oct-93	23	1.2	36	1.2
Nov-93	19	1.0	26	0.6
Aug-94	21	1.0	33	1.3

3.3.2.2. *Trichodina*

INTRODUCTION

There are several groups of protozoan ectoparasites that are known to cause mucus hypersecretion of the skin of fish. These include the genera *Trichodina, Chilodonella* and *Costia*. Both *Trichodina* and *Chilodonella* are ciliates, whereas *Costia* is a flagellate. These parasites typically cause the formation of a uniform mucus secretion on the trunk of the fish and can cause discolouration of the body. Microscopical investigation is frequently used, as in this investigation, to identify the main organisms responsible for ciliate - and flagellate - induced infection.

Trichodiniasis was formerly known as cyclochaetiasis with the parasite *Trichodina* in some cases being referred to as *Cyclochaeta*. Some confusion did exist over the nomenclature of this parasite, Van Duijn (1973), however, recommended that it be referred to as *Trichodina* rather than *Cyclochaeta.*

Cells of *Trichodina* spp. have a circular configuration with rings of cilia and a whorl of intracytoplasmic plates that are involved in cell adhesion. The cells usually have a diameter of 40 and 50μm (0.04-0.05mm), (Kudo, 1966) although it is not unusual for specimens of less than 10μm to occur. Reproduction is by simple division and occurs

on the host (Bauer and Nikolskaya, 1957). Transmission is by direct contact with the free - swimming organism in the water, (Kreier, 1978).

The parasite possesses a number of specialised attachment plates (see **Figures 3.60 - 3.63**). These plates provide the basis of a powerful sucker which permits adherence to the integument of the fish. *Trichodina* is found on most cyprinidae, gasterostidae, salmonidae and anguillidae Van Duijn (1973). Numerous studies, but most notably that of Chappell (1969), have reported 100% prevalence of *Trichodina* infection on sticklebacks. More importantly, they have confirmed the dominance of one specific species, namely, *Trichodina megamicronucleata*.

RESULTS

The presence of the ciliate, *T. megamicronucleata*, was noted for sticklebacks at sites B and E for the sampling period covered. This work was conducted on a purely qualitative basis since it was physically not practical to attempt to count individual ciliates. Results have been recorded using an arbitrary scale, based on intensity in **Table 3.5**. Every fish examined from both sites was infected with *Trichodina*.

Table 3.5: Presence and absence of the ciliate, *Trichodina megamicronucleata* on the integument of sticklebacks sampled from both sites B and E.

Sample date	Site B	Site E
Dec-92	+ +	+ +
Jan-93	+ + +	+ + +
Feb-93	+ + +	+ + +
Mar-93	+ + + +	+ + + +
May-93	+ + + +	+ + + +
Jun-93	+ + + +	+ + +
Sep-93	+ +	+ +
Oct-93	+ +	+ +
Nov-93	+ + +	+ + +
Aug-94	+ + +	+ + +

Key to Table 3.5:-

+ + + +	= Abundant
+ + +	= Frequent
+ +	= Occasional
+	= Rare

Table 3.5: shows that *T. megamicronucleata* was present, in some considerable numbers, at both sampling sites. *T. megamicronucleata* infection was very uniform throughout the period sampled and at each of the sampling sites. The level of ciliate infection at site B and E was noted to be probably highest during the period between March - May 1993, with decreases being seen to occur in the level of ciliate infection during the later months at both sites. Overall there was little difference in apparent infection levels at the two sites.

3.3.2.3 *Gyrodactylus*

INTRODUCTION

Gyrodactylids are monogenean (phylum Platyhelminthes, class Trematoda, order Monogenea and Super-family Gyrodactyloidea) ectoparasites of the gill and integument and can cause significant mortality in fishes if infection is high. Skin discoloration, fin abnormalities, desquamation and haemorrhage are known to occur in seriously infected fishes. The general morphology of these parasites has been characterised by Van Duijn (1973).

Intensity and mean intensity was calculated for *G. arcuatus* infection (**Figures 3.71** and **3.72**). There was considerable month - to - month variation in mean intensity at both sites. At site B values ranged from a low of 0.4 in August 1994 to a high of 28.5 in March 1993. At site E values ranged from a low of 1.2 in October 1993 to a high of 25.4 in June 1993.

Gyrodactylus is a live-bearing parasite and the developing embryos and juveniles can be seen clearly within the "parental" organism. Additionally, the embryos are parthenogenetic and can produce new embryos before they themselves are born. Such parthenogenetic qualities in the embryo are referred to as **paedogenesis** and this is a feature common to all gyrodactylid species (**Figure 3.64**). This feature, the paedogenesis, and presence of several embryos in the parasite can also be used to distinguish species in the genus *Gyrodactylus* from species in the genus *Dactylogyrus* which are egg - producing monogeneans which do not harbour embryos.

The new - born parasites have no cilia and closely resemble the adult and they have an opisthaptor with a pair of anchors and 16 marginal hooklets (**Figure 3.65**). The young attach to the skin or gill of the same host as the parent worm. Having no cilia , they presumably transfer to other host fish when there is physical contact between the hosts (Marquardt and Demaree Jr, 1985).

RESULTS

Figures 3.66 and 3.67 show *Gyrodactylus arcuatus* on the fins of the three - spined sticklebacks while the **Figure 3.68** illustrates a detached individual.

The prevalence of the *Gyrodactylus* infection varied between 22 and 100% at site B and between 38.1 and 100% at site E (**Figures 3.69 and 3.70**). Because the number of parasite in a single monthly sample from one site could approach 1000, it was not practical to make a detailed, microscopical identification of every worm. All of those individuals that were examined in this way, however, proved to be *G. arcuatus*. There was no evidence of other monogenean species, for example *Dactylogyrus vastator* or *Dactylogyrus extensus*, being present in any of the samples.

Intensity and mean intensity was calculated for *G. arcuatus* infection (**Figures 3.71** and **3.72**). There was considerable month - to - month variation in mean intensity at all site. At site B values ranged from a low of 0.4 in August 1994 to a high of 28.5 in March

1993. At site E values ranged from a low of 1.2 in October 1993 to a high of 25.4 in June 1993.

3.3.2.4 *Proteocephalus*

INTRODUCTION

The order Proteocephala is composed of about 30 genera of cestodes, all endoparasitic in fishes, amphibians and reptiles as adults (Yamaguti, 1959; Freze, 1965). The adult and the two metacestode stages, procercoids and plerocercoids, all have scolices with four suckers and in some species an additional fifth apical sucker is present as well. A distinctive morphological feature of adult proteocephalans is the lateral location of the vitellaria in mature proglottids. Most proteocephalans belong to the genus *Proteocephalus*.

Until recently, the life cycles of proteocephalids have been somewhat of a mystery because, at least in some species, there appeared to be one stage missing in the life cycle and possibly in all species the definitive fish host acts additionally as an intermediate host (Fischer and Freeman, 1969). The gravid proglottids are passed in host faeces; they rupture upon contact with water, releasing embryonated eggs. Eggs are ingested by crustaceans including *Cyclops* sp. and procercoids develop in the hemocoele. It is the next stage in the life cycle about which confusion has arisen. Because plerocercoids are frequently found in the gut of the definitive fish hosts it has been generally assumed that in the life cycle either (a) no second intermediate host was required and development from procercoid (ingested in a copepod) to plerocercoid to adult could take place in the definitive fish host or (b) a second intermediate host containing (encysted) plerocercoids was involved (Fischer and Freeman, 1969).

RESULTS

When the intestinal lumens of sticklebacks were examined for *Proteocephalus* infections, it was apparent that both gravid adult worms and non - gravid juvenile (post - plerocercoids) were both present. It was noted that in most cases the former were present in the posterior half of the intestine, while the latter were found in the anterior half. In analyses of intensity and mean intensity of *Proteocephalus* infection juvenile and adult numbers have been added together. Analysis of prevalence has considered the two developmental stages separately.

The occurrence of the cestode, *Proteocephalus filicollis*, was recorded at both sites B and E (**Figures 3.73 and 3.74**).

Overall, the prevalences of infection (**Figures 3.75** and **3.76**) were higher at site B than at site E with adult prevalence reaching 57.8% in February 1993 at site B, whereas the highest adult prevalence at site E was only 17.7% in March 1993. Parasites were present in fish in every sample at site B, while at site E no parasites could be found in May, June or September 1993.

Analysis of intensity and particularly of mean intensity (**Figures 3.77** and **3.78**) show that site B sticklebacks had a far higher level of infection than those at site E. Mean intensities peaked at 5.7 in March 1993 at site B, while the highest level at site E was 1 in December 1992. Both prevalence and mean intensity data suggest a greater force of infection in the winter months, December to March, than in the rest of the year. The differential analyses of adult and junenile prevalences (**Figures 3.75** and **3.76**) show that at site B adult prevalence exceeded that of juveniles in all months apart from June and September 1993.

3.3.2..5. *Glugea*

INTRODUCTION

Protozoan parasites in the genus *Glugea* are a major cause of "pimple" disease of fishes. In sticklebacks, two species of *Glugea* are most common, chiefly, *Glugea anomala* and *Glugea pseudotumefaciens*. *G. anomala* is typically found to infect the skin and gill whereas *G. pseudotumefaciens* causes protrusion of the eye from the orbits (exophthalmos). The cysts themselves can be between 2-6mm in diameter and are widely distributed across the skin with the cyst being embeded in the trunk musculature. Each cyst is "spore-rich" containing very large numbers of sub - spherical spores of length 4.5-5.0 μm and width 3.0-4.0μm (Van Duijn, 1973). *Glugea* is a member of the sub-class, the Neosporidia and actively produces sporozoites throughout its life-cycle.

Only one species of *Glugea*, that being *Glugea anomala*, was isolated from Wandle sticklebacks. The other common species, *Glugea pseudotumefaciens* was not found in any of the Wandle sticklebacks examined during this investigation.

The spores are ingested and the polar filament injects the sporont which is a uninucleate vacuolar cell into the cells of the gut and from here the infection may extend to other tissues and organs (Marquardt and Demaree Jr, 1985). The sporoplasm transforms into a trophozoite and undergoes merogony. There is more than one merogonous generation, and then the merozoites reinvade cells to form sporonts. Each sporont gives rise to only a single spore. Spores may be formed as early as 48 hr after infection (Marquardt and Demaree Jr, 1985).

RESULTS

Glugea anomala was present at both sites B and E (**Figures 3.79 and 3.80 and 3.81**). The prevalence and intensity of infection by *Glugea anomala* was recorded for both sites B and E. It must be noted that in all this epidemiological analysis it is not individual protozoan cells which are being monitored but macroscopic cysts.

Prevalence of *Glugea* infection at site B was found to be very low and parasites were present only in 4 of the 10 months sampled. Furthermore, the maximum prevalence of infection recorded was 15.3% in August 1994 (**Figure 3.82**). In contrast, site E was much more highly infected by *Glugea*, with the prevalence ranging between 7.1 - 70.8% over the 10 months sampled and parasites being present in every sample. (**Figure 3.83**).

This disparity in infection level at the two sites was just as striking when intensity measures were analysed. **Figure 3.84** shows that peak mean intensity of cysts was only 0.2 in August 1994 at site B and zero or much lower in all other months. Only in one month (May 1993) did the mean intensity at site E (**Figure 3.85**) drop lower than 0.2, while the maximum value was 0.7 in December 1992.

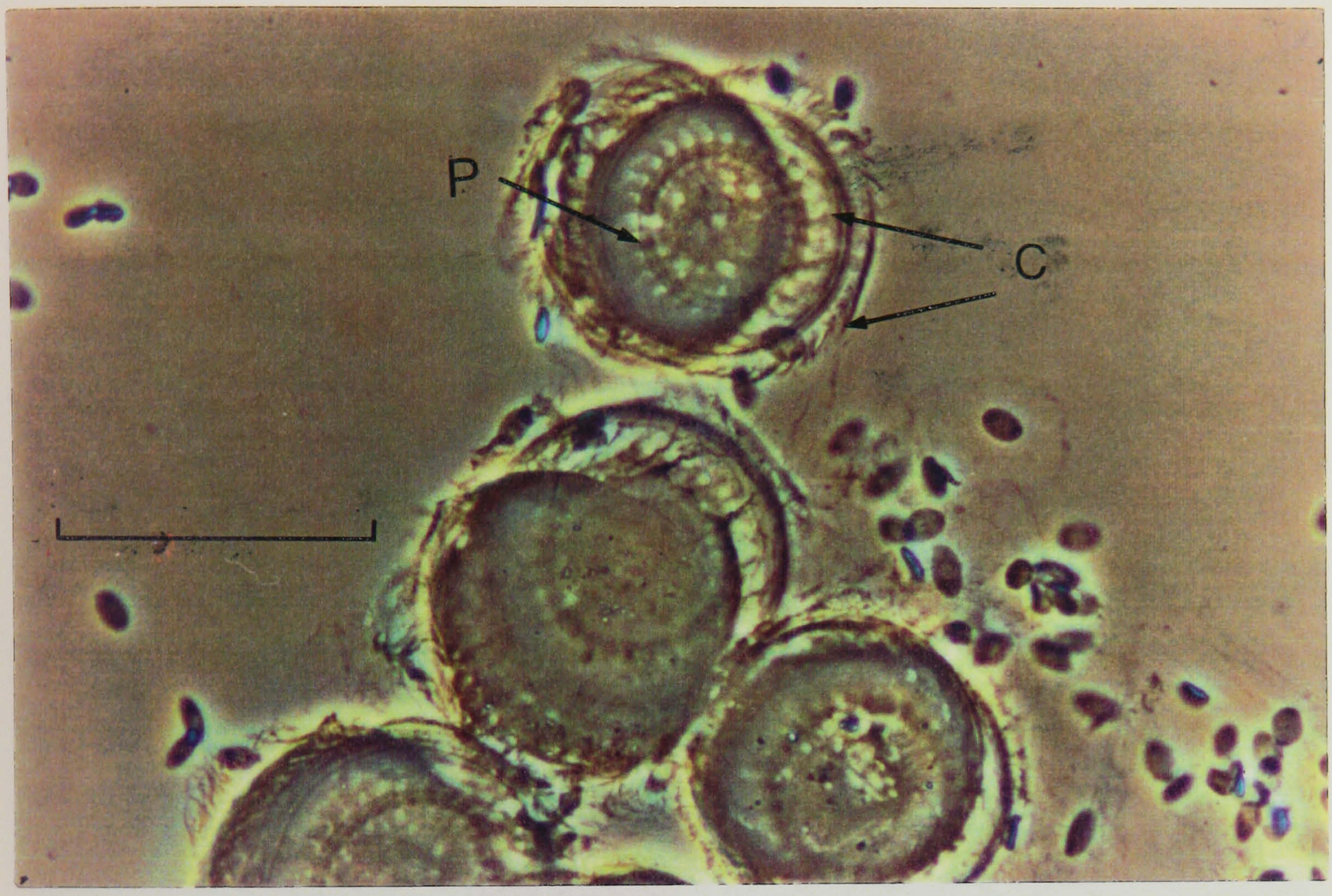


Figure 3.60: Phase contrast micrograph of *Trichodina megamicronucleata* isolated from the caudal and pectoral fins of the stickleback. Notice the concentric nature of the ciliate, the presence of numerous cilia and the "teeth-like"plate structures that secure parasite attachment to the integument and fins of the fish. (C = cilia; P = plates) scale bar = 100μm.

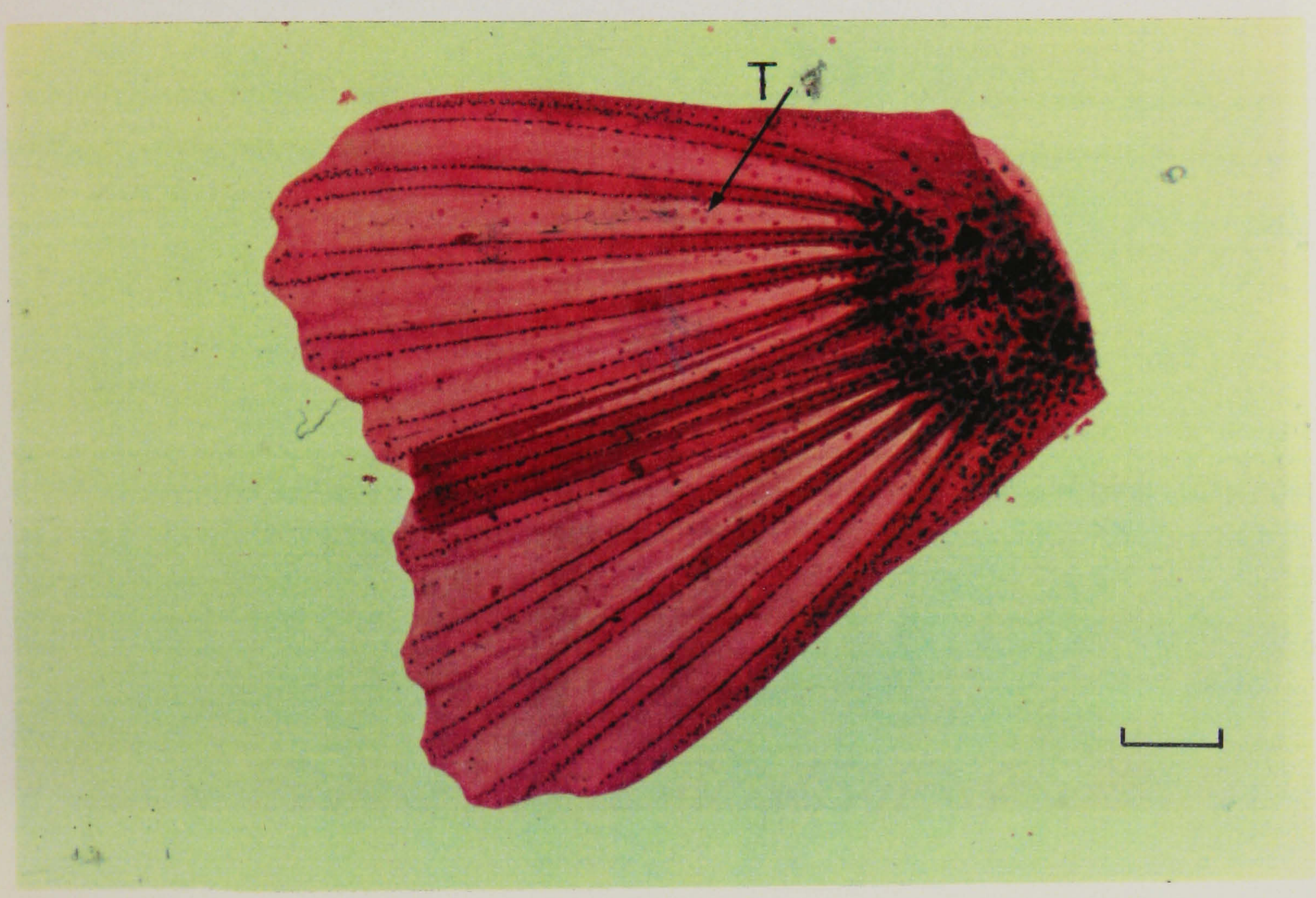


Figure 3.61: An infection with the ciliate *Trichodina megamicronucleata* is evident here on the caudal fin of a stickleback. Specimen stained with Borax - carmine. (T = *Trichodina*), scale bar = 1mm.

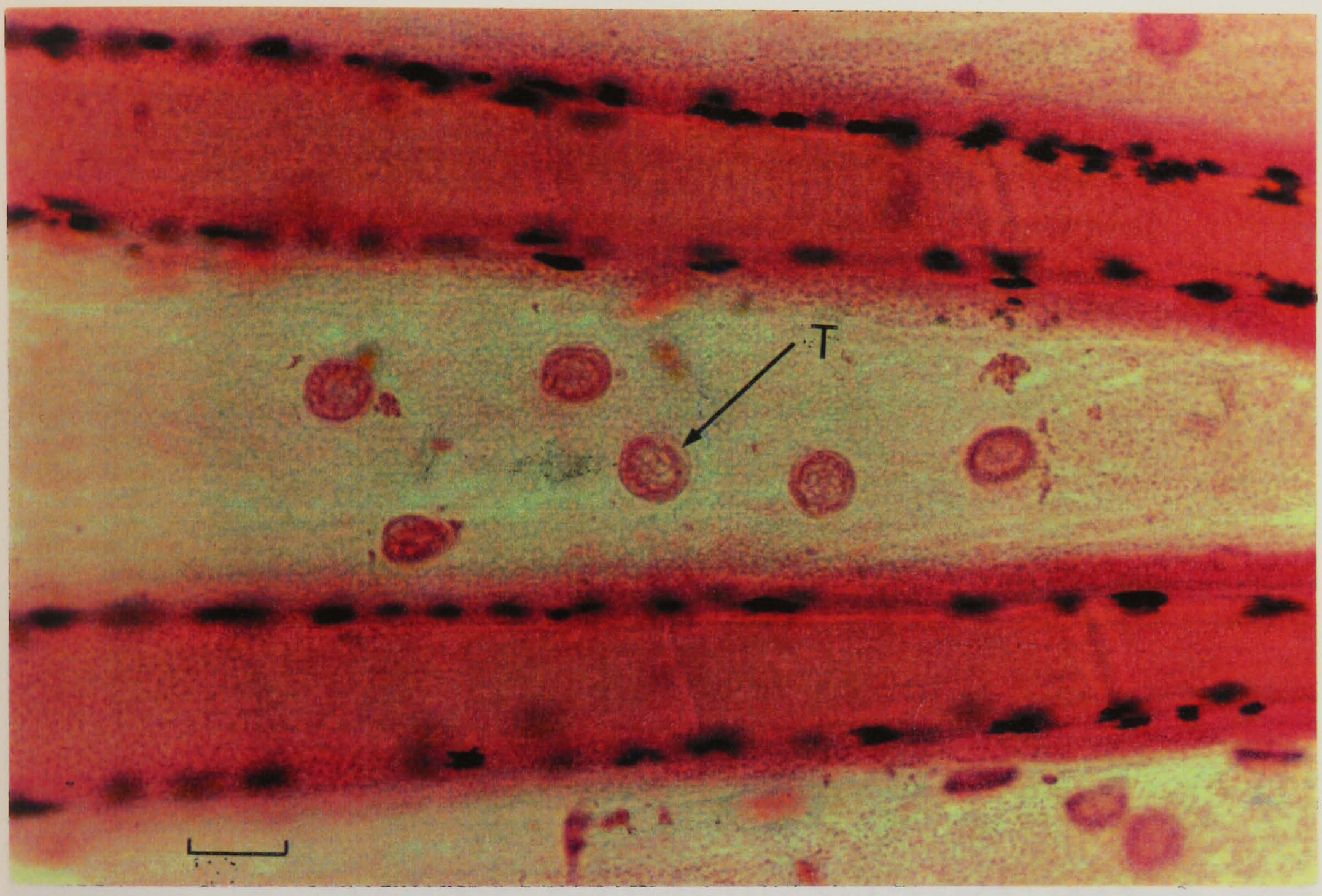


Figure 3.62: A higher magnification reveals the circular morphology of the ciliate *Trichodina megamicronucleata* between the fin-rays of stickleback caudal fin. Specimen stained with Borax - carmine. (T = *Trichodina*), scale bar = 100μm.

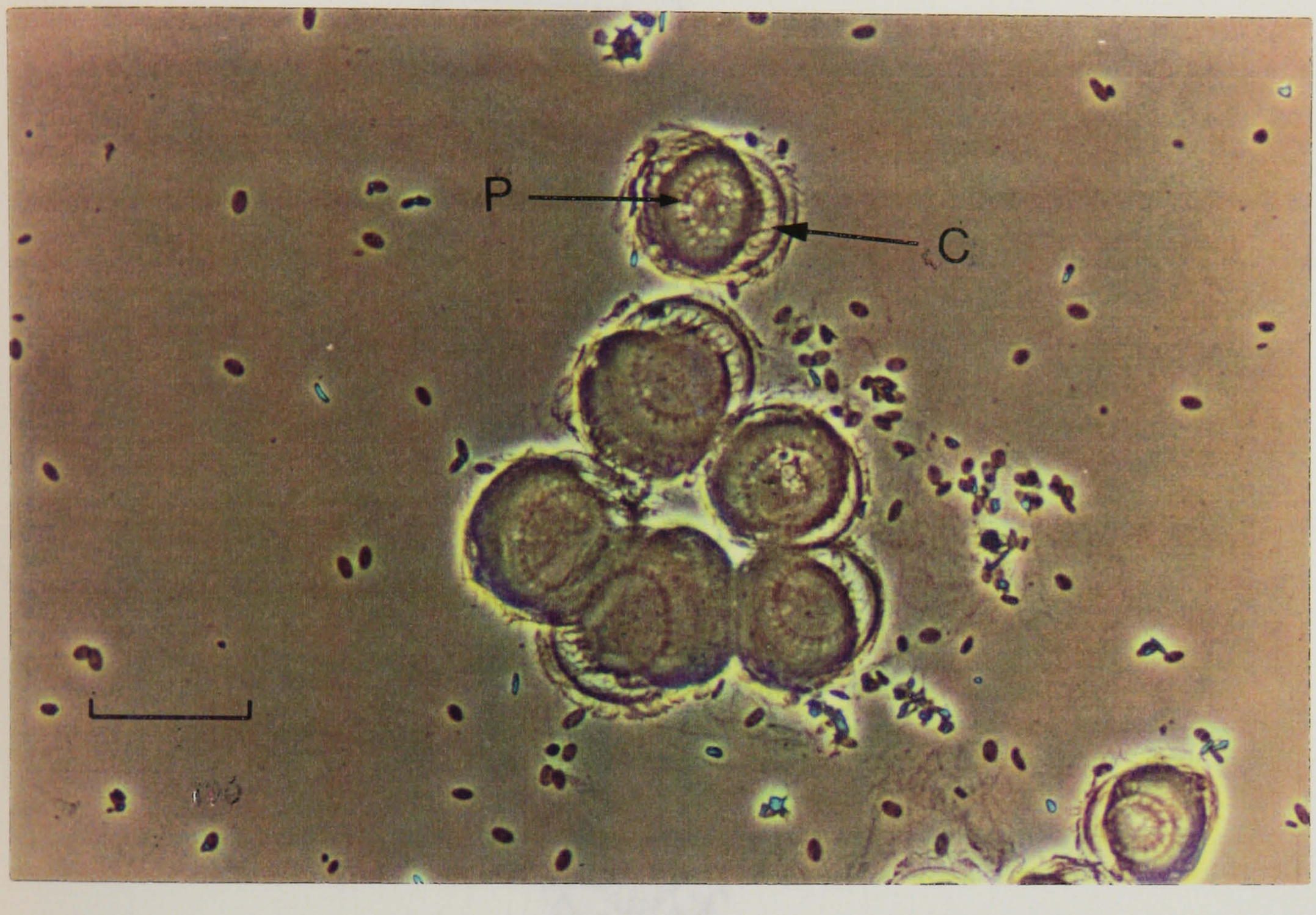


Figure 3.63: Phase contrast micrograph of a group of detached *Trichodina megamicronucleata*, isolated from the fins (caudal and pectoral) of the stickleback. (C = cilia; P = plate), scale bar = 100μm.

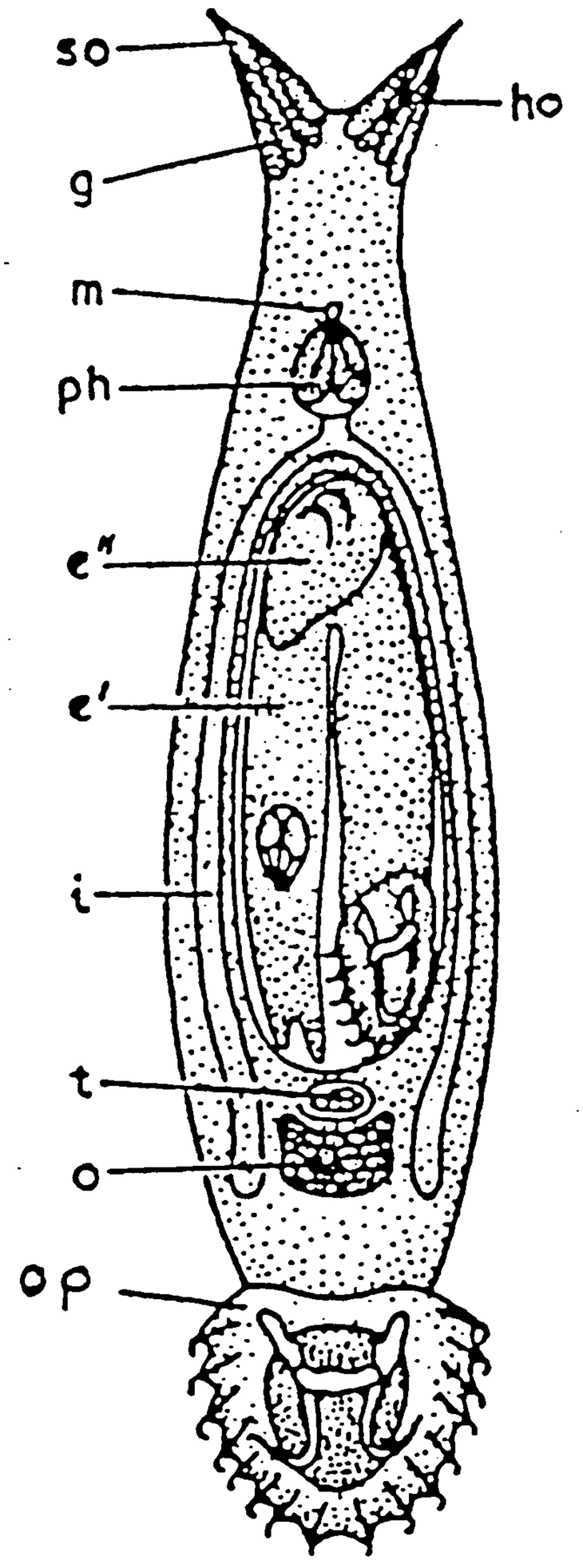


Figure 3.64: A schematised representation of the structure of the monogenean, *Gyrodactylus arcuatus* from Van Duijn (1973) (see next page to the key of the **Figure 3.64**).

Key to Figure 3.64:-

ho: head organ (lappet)

g: gland

so: sense organ

m: mouth

ph: pharynx

e": embryo of the second generation

e': embryo of the first generation

i: intestine

o: ovary

t: testis

op: opisthaptor

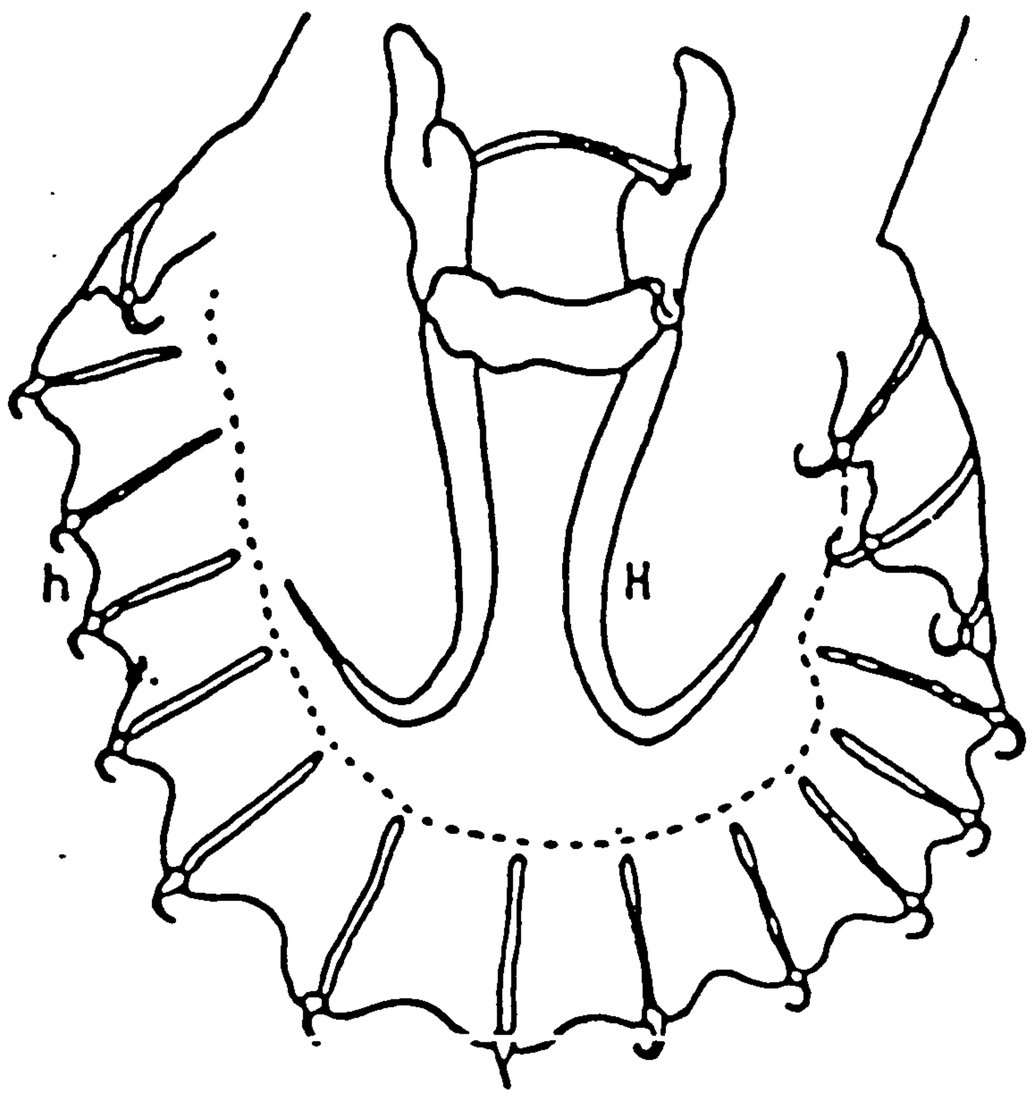


Figure 3.65: Diagramatic representation of the structure of the opisthaptor organ of the monogenean, *Gyrodactylus arcuatus* adapted from Van Duijn (1973). Note the position of the sixteen hooklets and the two major posterior hooks on the opisthaptor organ. [h = hooklets (16); H = attachement hooks (2)].

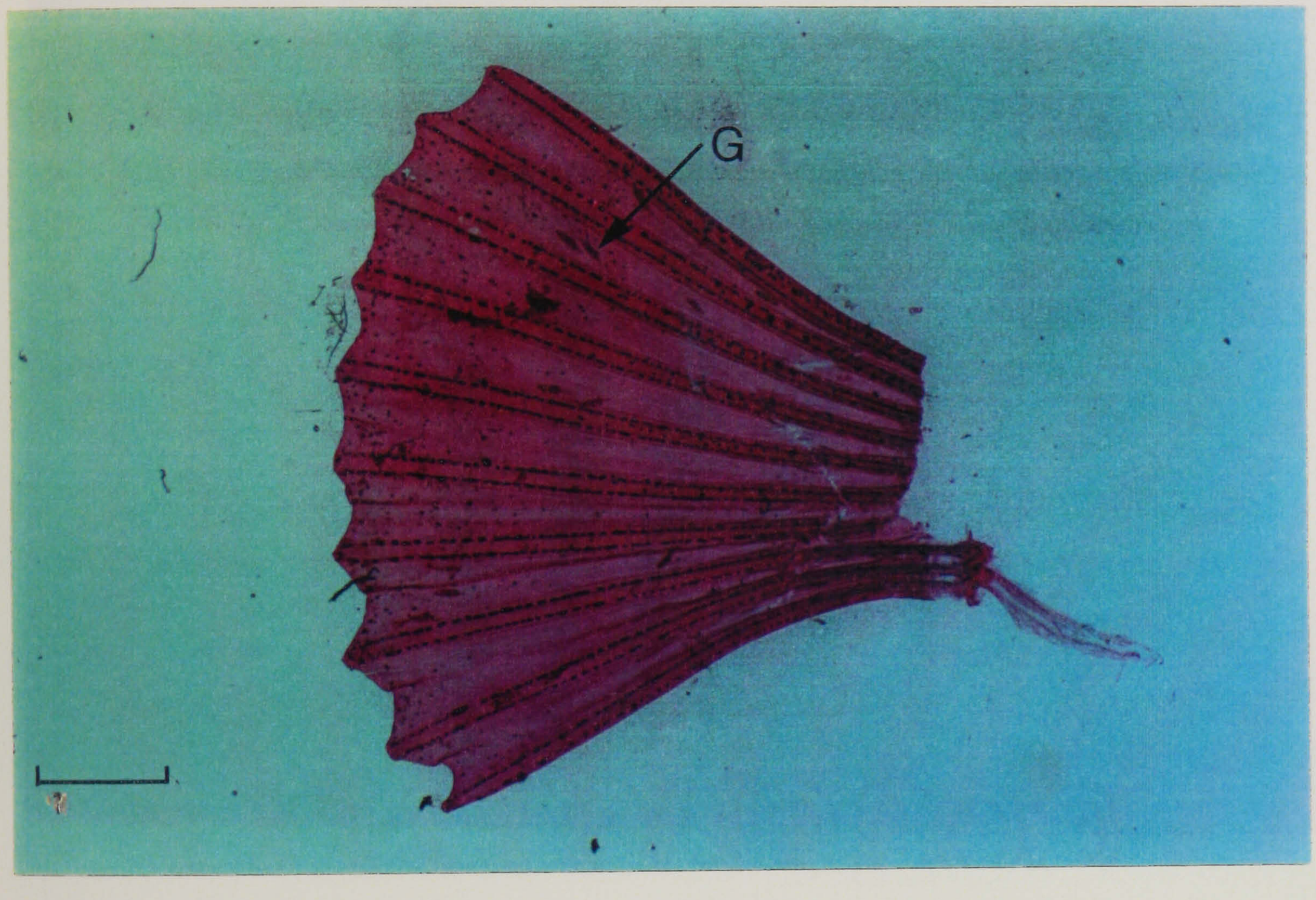


Figure 3.66: Specimens of the monogenean parasite, *Gyrodactylus arcuatus*, on the caudal fin of a stickleback. The specimen stained with Borax - carmine. (G - *Gyrodactylus*) scale bar = 1mm.

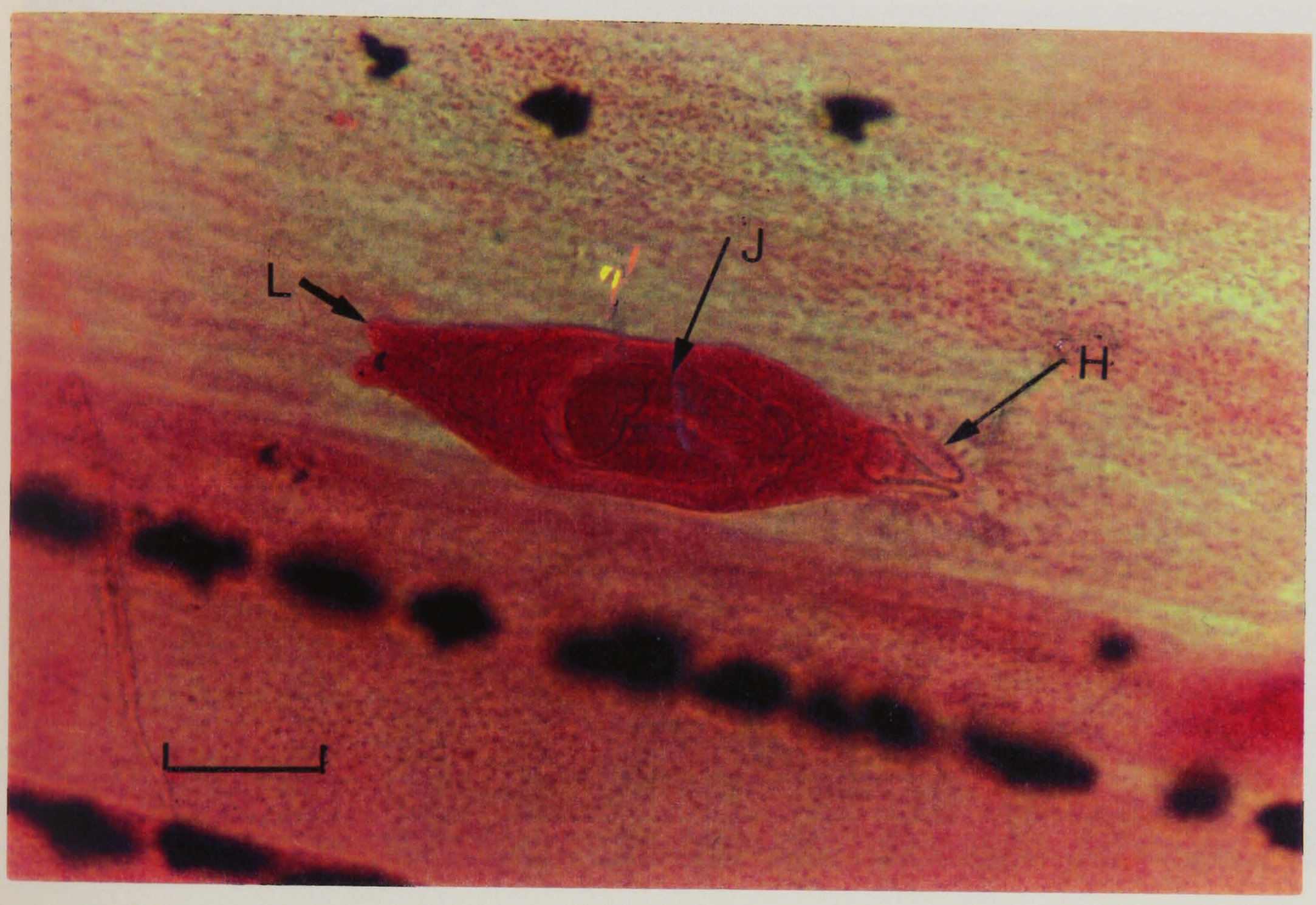


Figure 3.67: Specimen of *Gyrodactylus arcuatus* in the space between two fin rays on the caudal fin of a stickleback. The monogenean shows head lappets (L), enclosed developing juvenile (J) and a hooked opisthaptor (H). Specimen stained with Borax - carmine. (scale bar = 100μm).

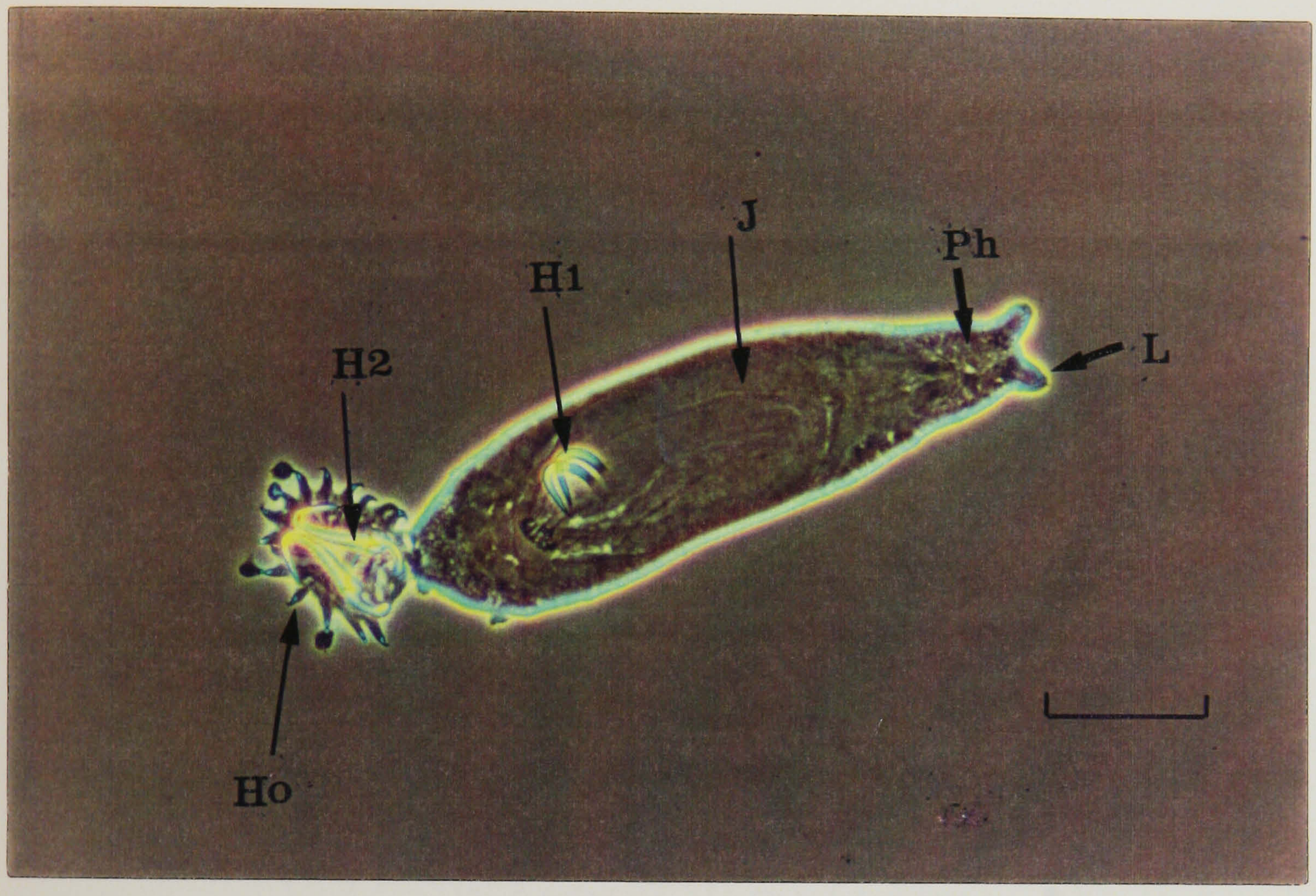


Figure 3.68 Phase contrast micrograph showing the main features of *Gyrodactylus arcuatus*. (L = lappet with sensory ending; Ph = pharynx; J = 1st generation juvenile; H1= opisthaptor hooks of enclosed juvenile; H2= opisthaptor hooks of adult; Ho = hooklets of adult). (scale bar = 100μm).

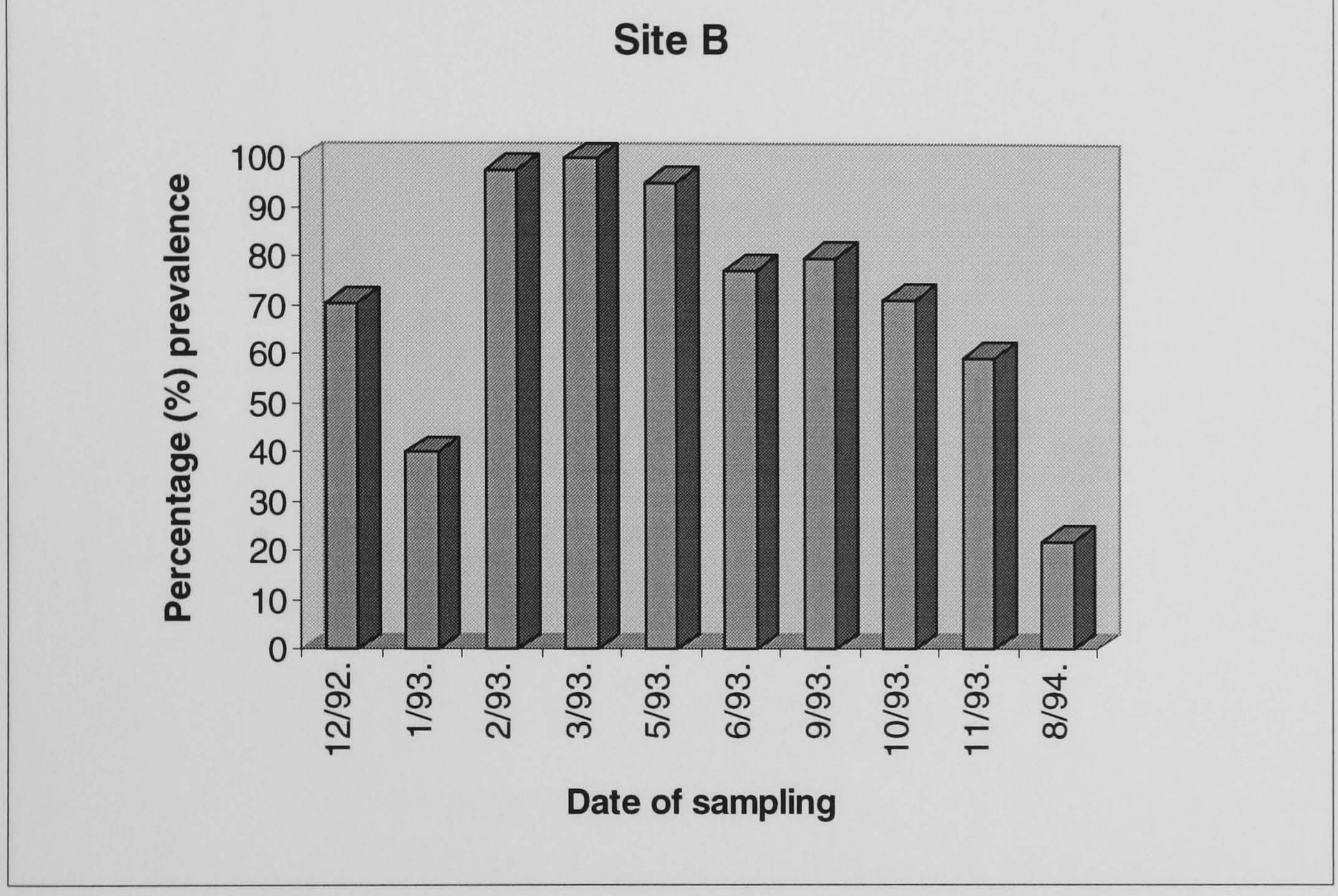


Figure 3.69: Prevalence of *Gyrodactylus arcuatus* infection in *G. aculeatus* at site B.

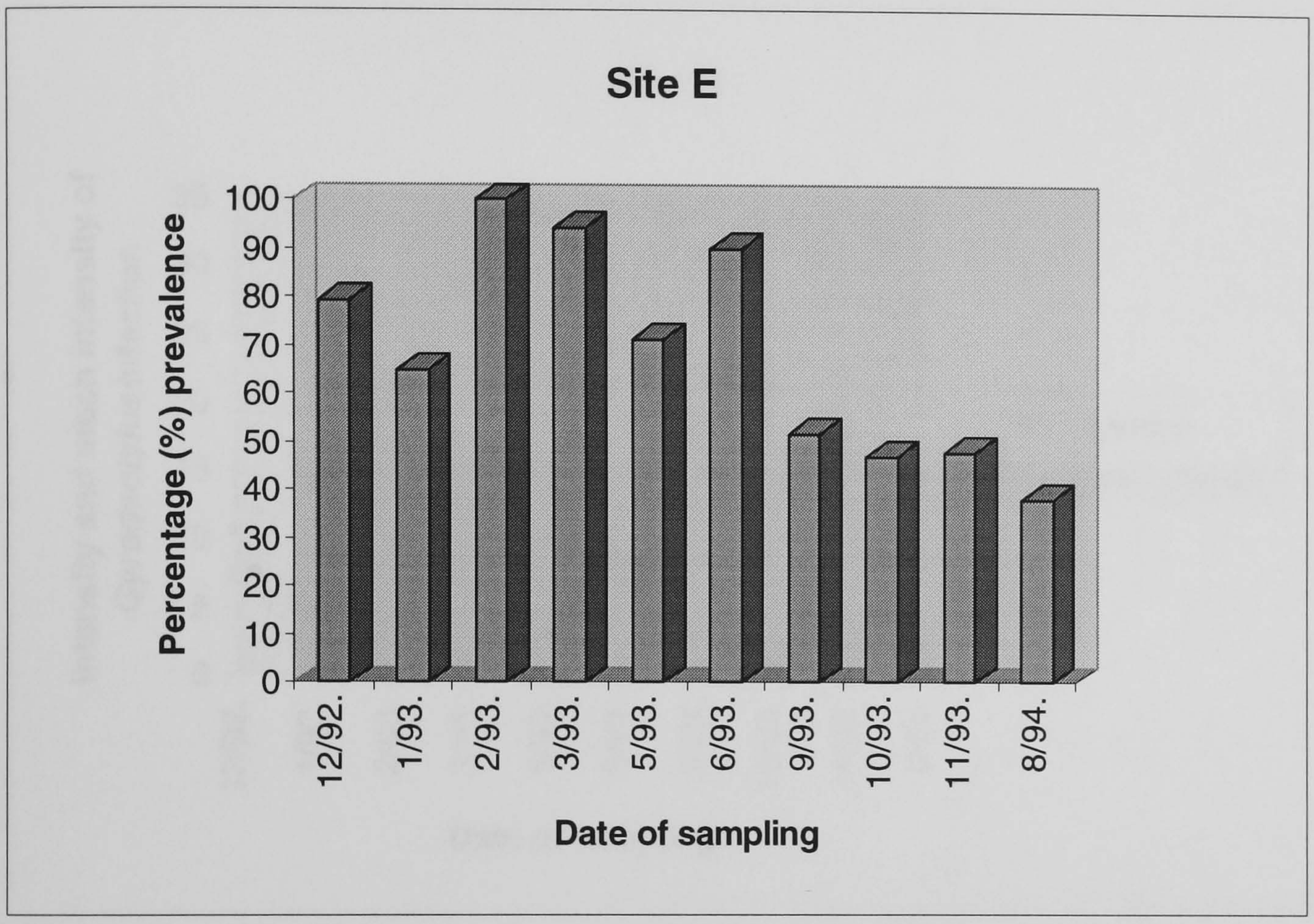


Figure 3.70: Prevalence of *Gyrodactylus arcuatus* infection in *G. aculeatus* at site E.

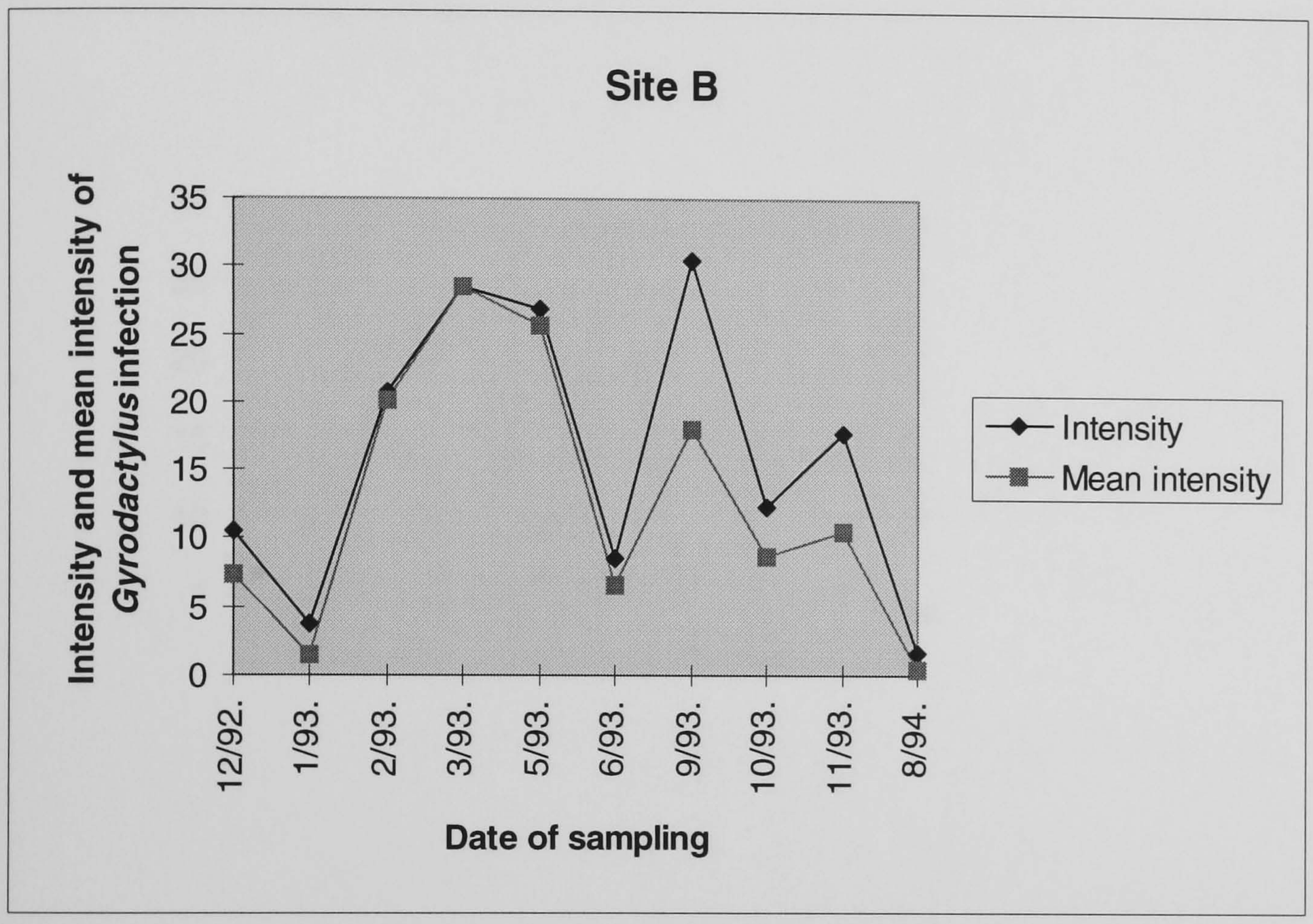


Figure 3.71: Intensity and mean intensity of *Gyrodactylus arcuatus* infection on *G. aculeatus* from site B during the period from December 1992 to August 1994.

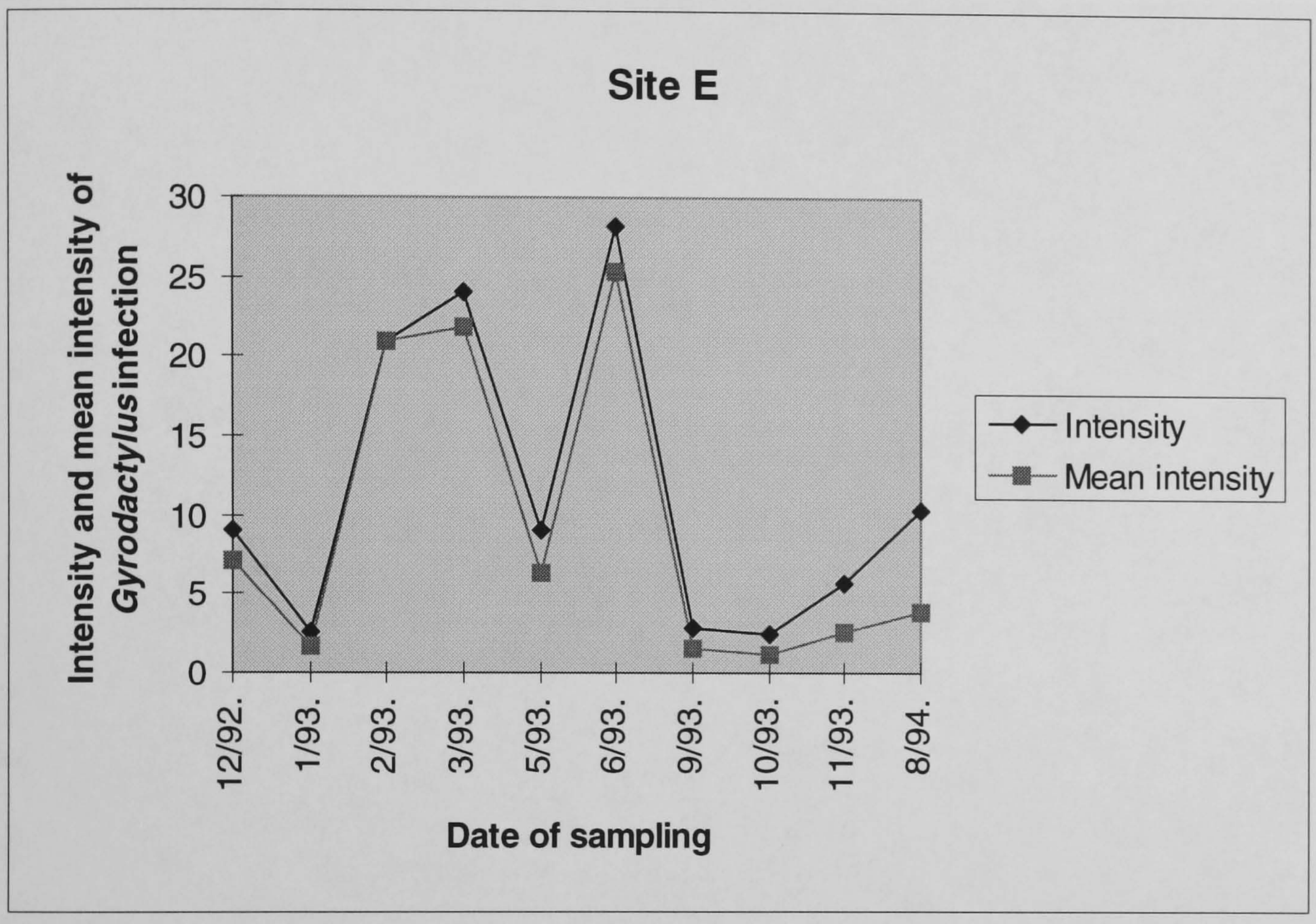


Figure 3.72: Intensity and mean intensity of *Gyrodactylus arcuatus* infection on *G. aculeatus* from site E during the period from December 1992 to August 1994.

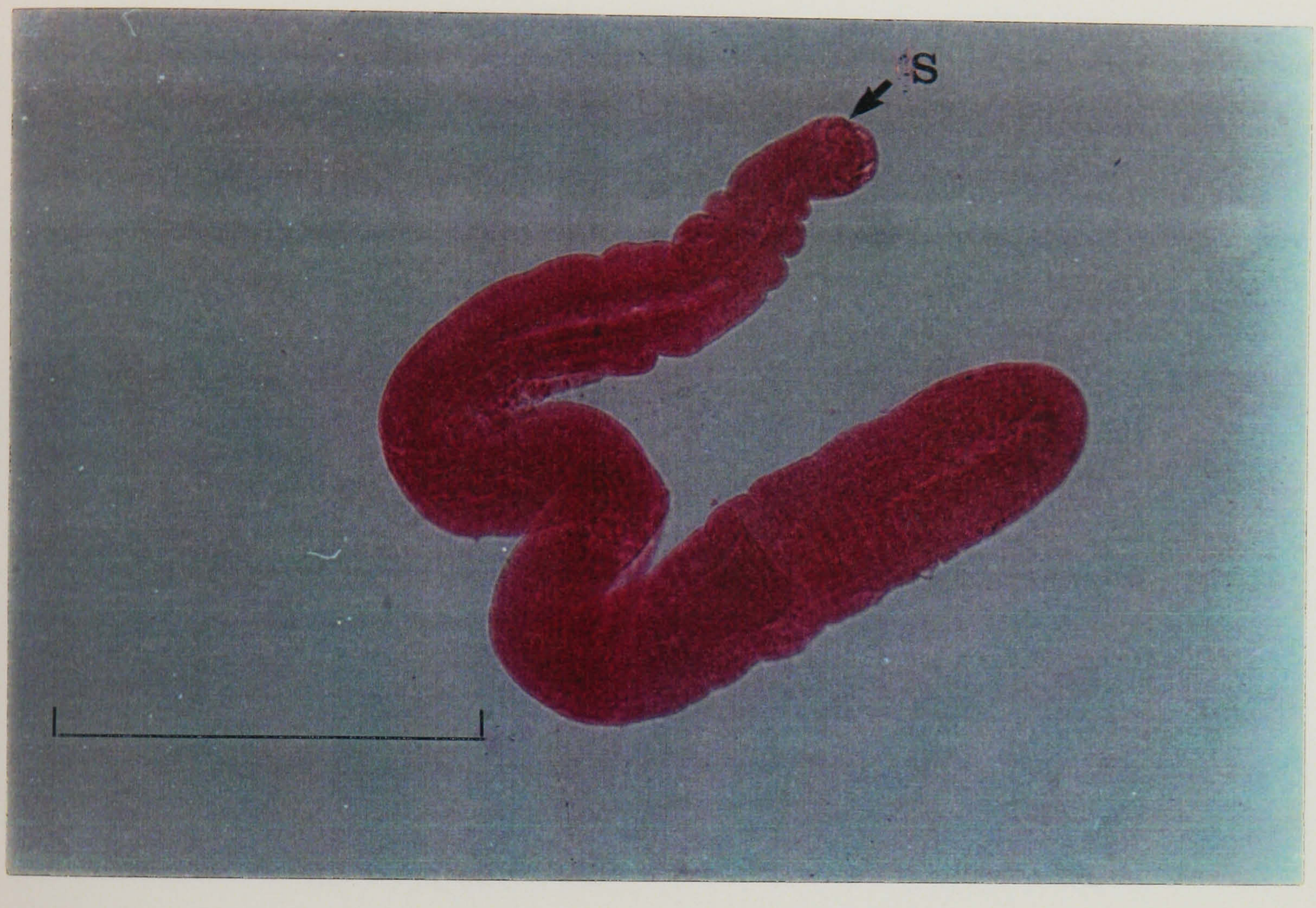


Figure 3.73: A whole mount micrograph of a juvenile stage of the cestode, *Proteocephalus filicollis*, isolated from anterior part of the intestinal lumen of the three-spined stickleback (*Gasterosteus aculeatus*). The parasite was fixed in 10% formalin solution and stained using Borax -carmine stain. (S = scolex) (scale bar = 1mm).

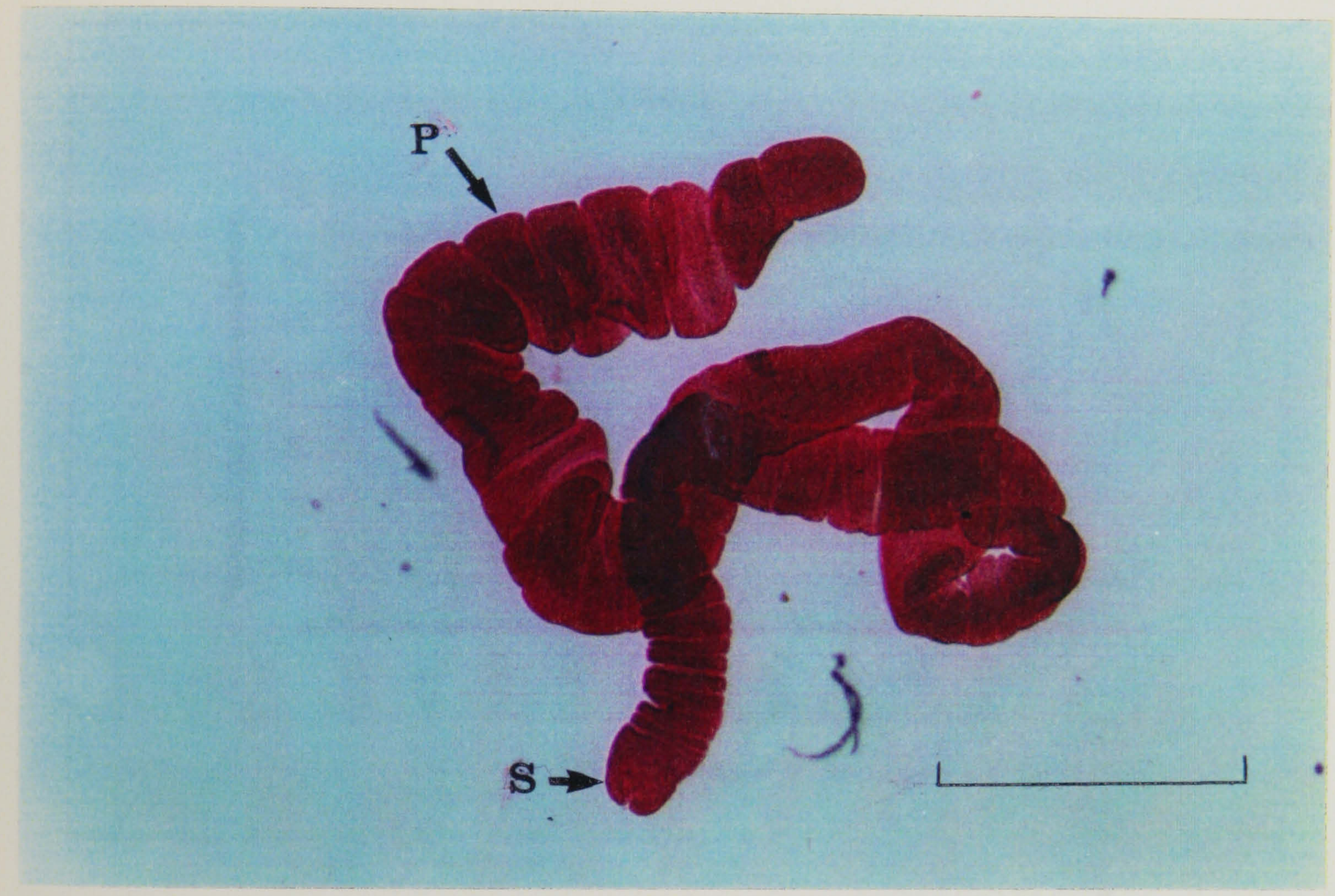


Figure 3.74: A whole mount micrograph of an adult cestode, *Proteocephalus filicollis*, isolated from the posterior part of the intestinal lumen of the three-spined stickleback (*Gasterosteus aculeatus*). The parasite was fixed in 10% formalin and stained in Borax-carmine stain.(S = scolex; P = proglottid) (scale bar = 1mm).

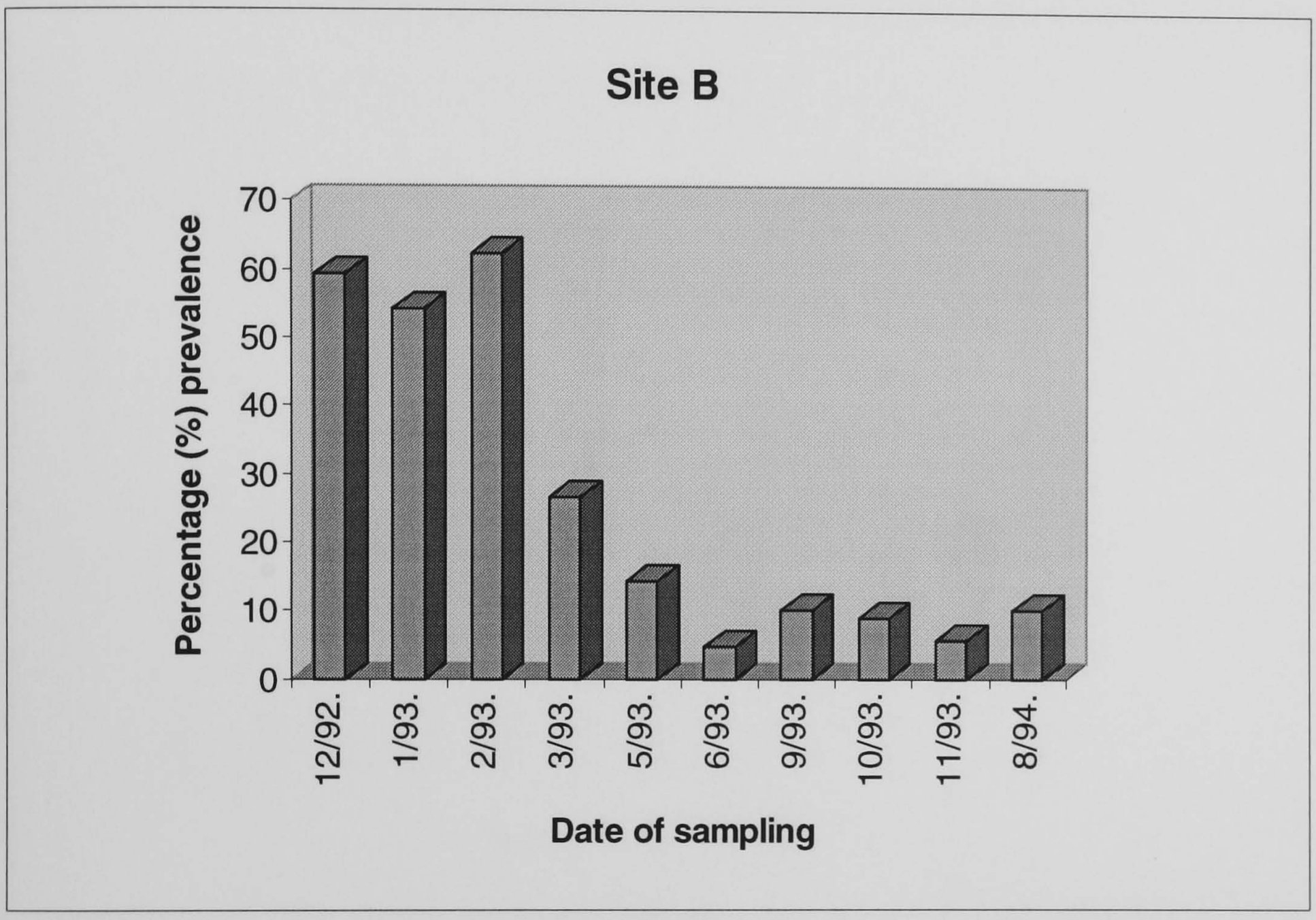


Figure 3.75: Prevalence of *Proteocephalus filicollis* infection in *G. aculeatus* at site B.

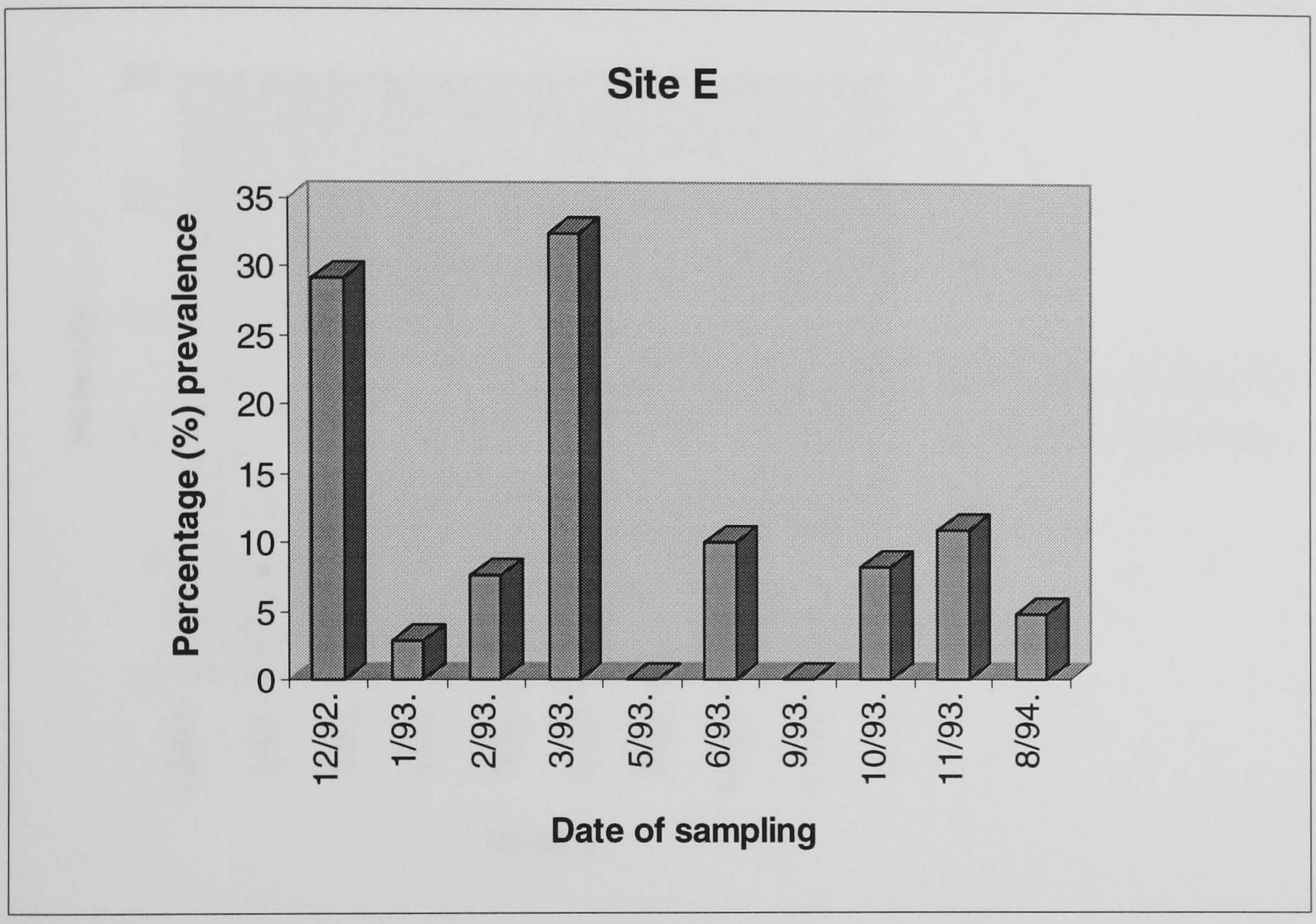


Figure 3.76: Prevalence of *Proteocephalus filicollis* infection in *G. aculeatus* at site E.

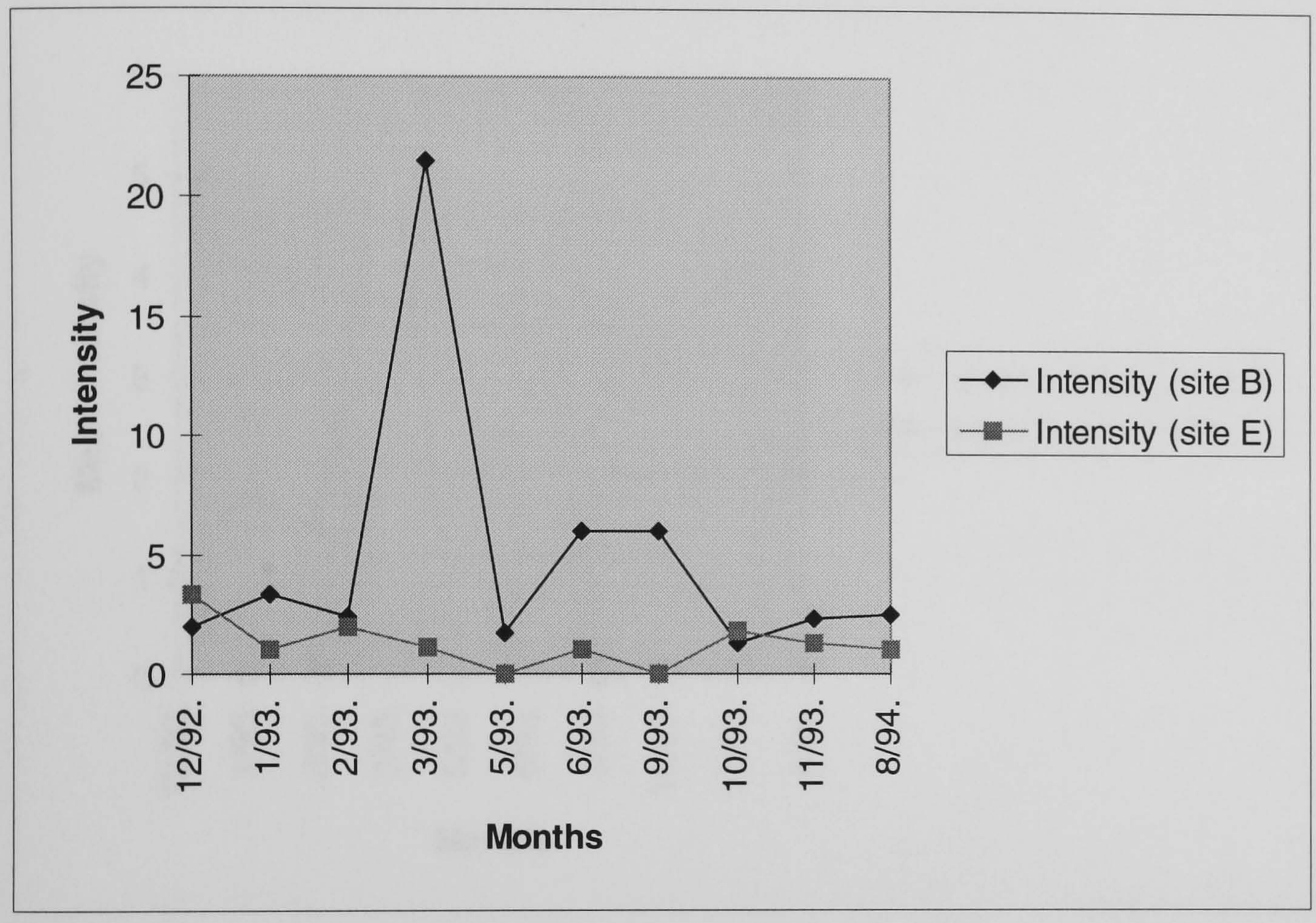


Figure 3.77: Intensity of *Proteocephalus filicollis* in *G. aculeatus* from both sites B and E during December 1992 to August 1994.

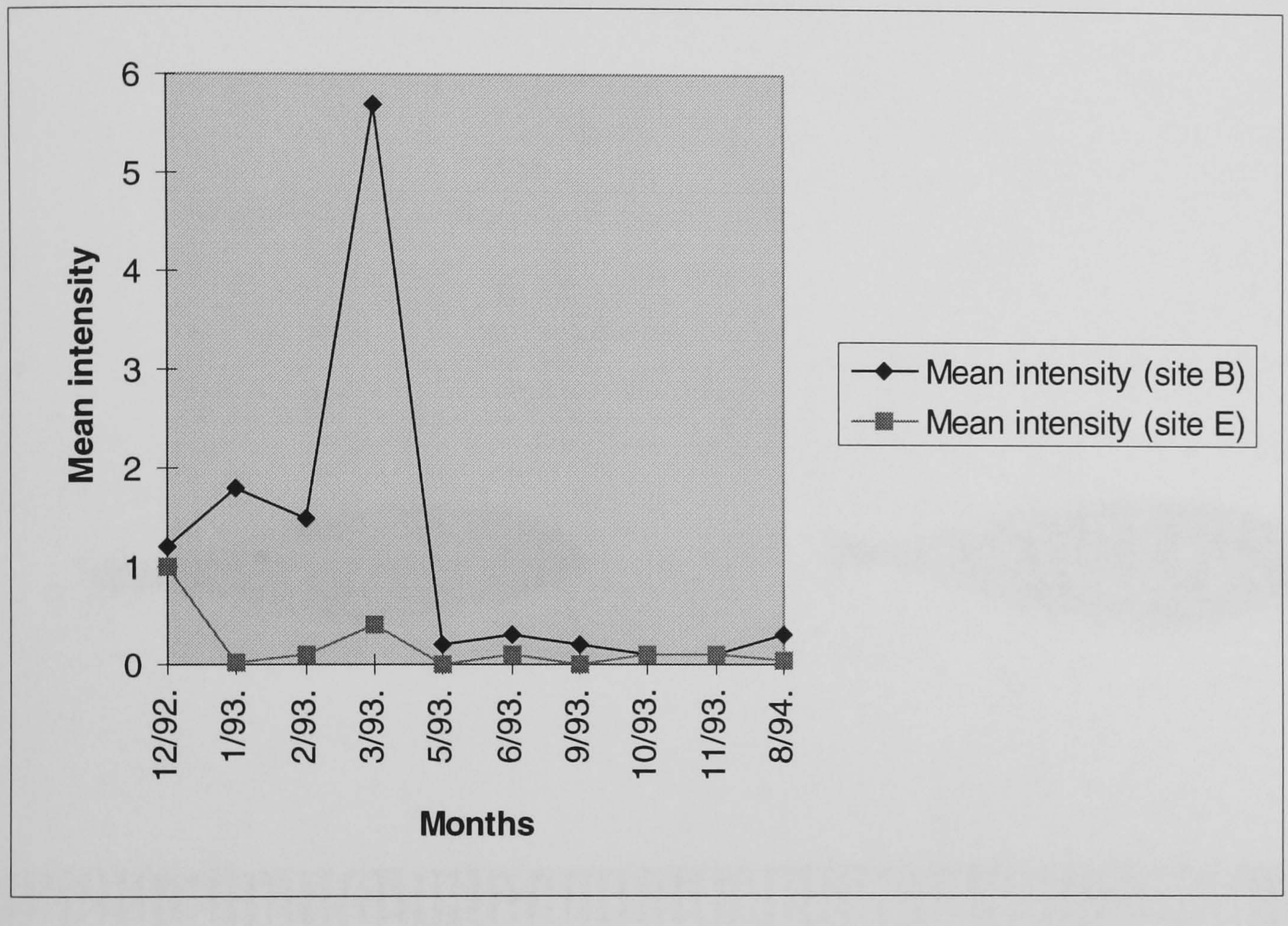


Figure 3.78: Mean intensity of *Proteocephalus filicollis* infection in *G. aculeatus* from both sites B and E during the sample period from December 1992 to August 1994.

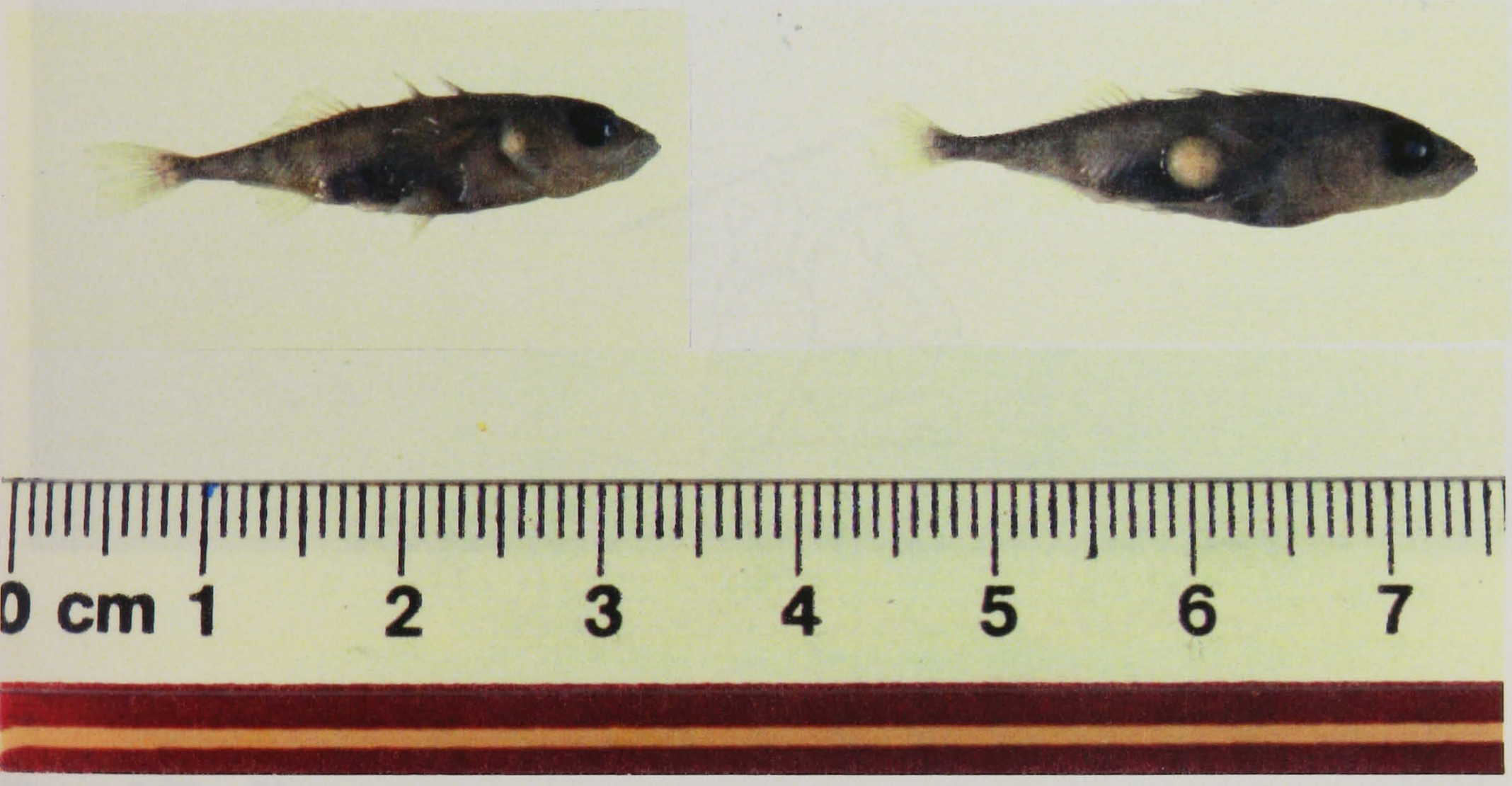


Figure 3.79: Three - spined sticklebacks showing *Glugea anomala* cysts.

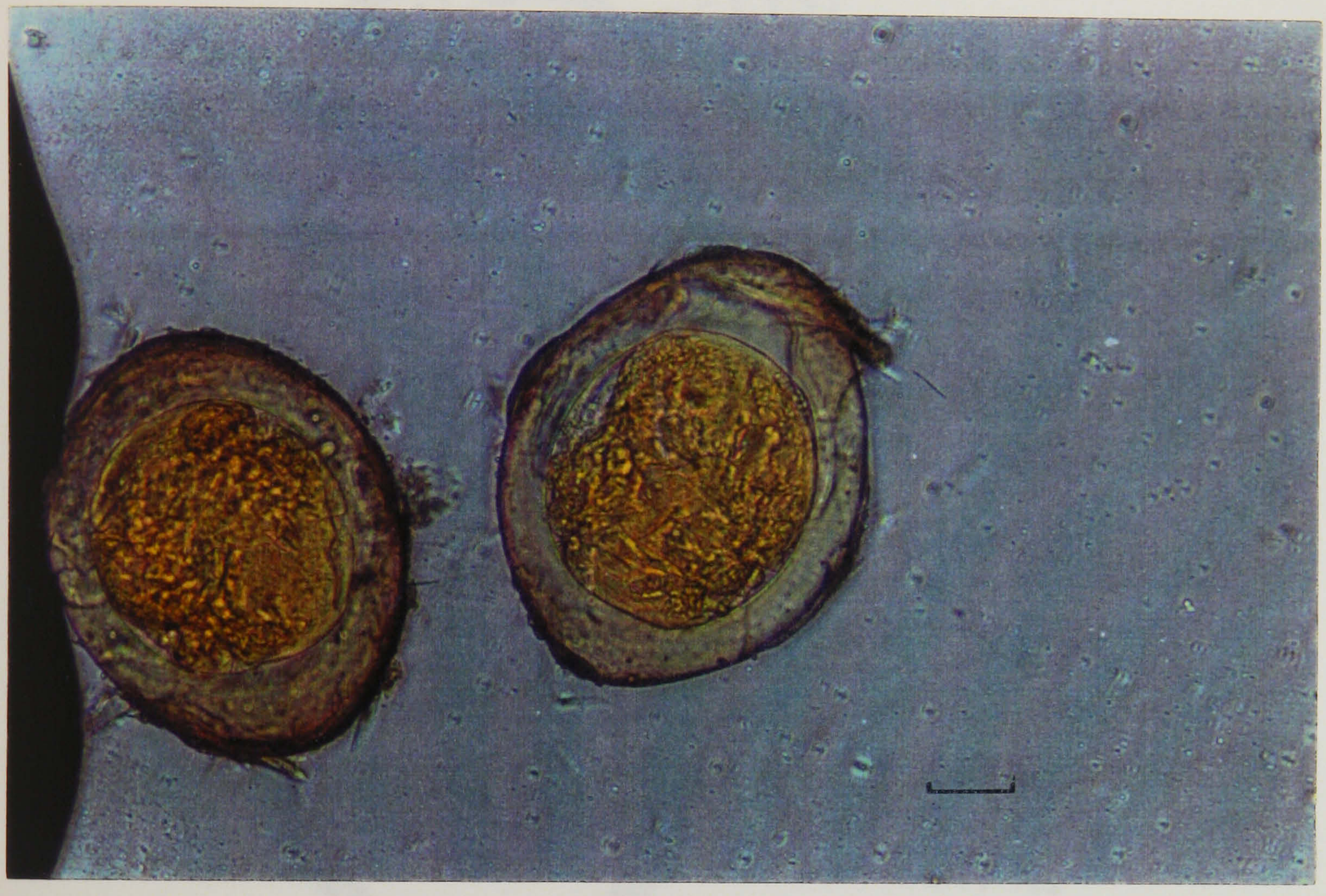


Figure 3.80: Phase contrast micrographs of entire cysts of *Glugea anomala* isolated from the subcutaneous layer of the skin of the stickleback. Cysts such as these were isolated from a number of subcutaneous sites of sticklebacks plus examples from gills and the opercular plates. **Figure 3.81** provides a schematic representation of the locations of the cysts in sticklebacks (Van Duijn, 1973). (scale bar = 100μm).

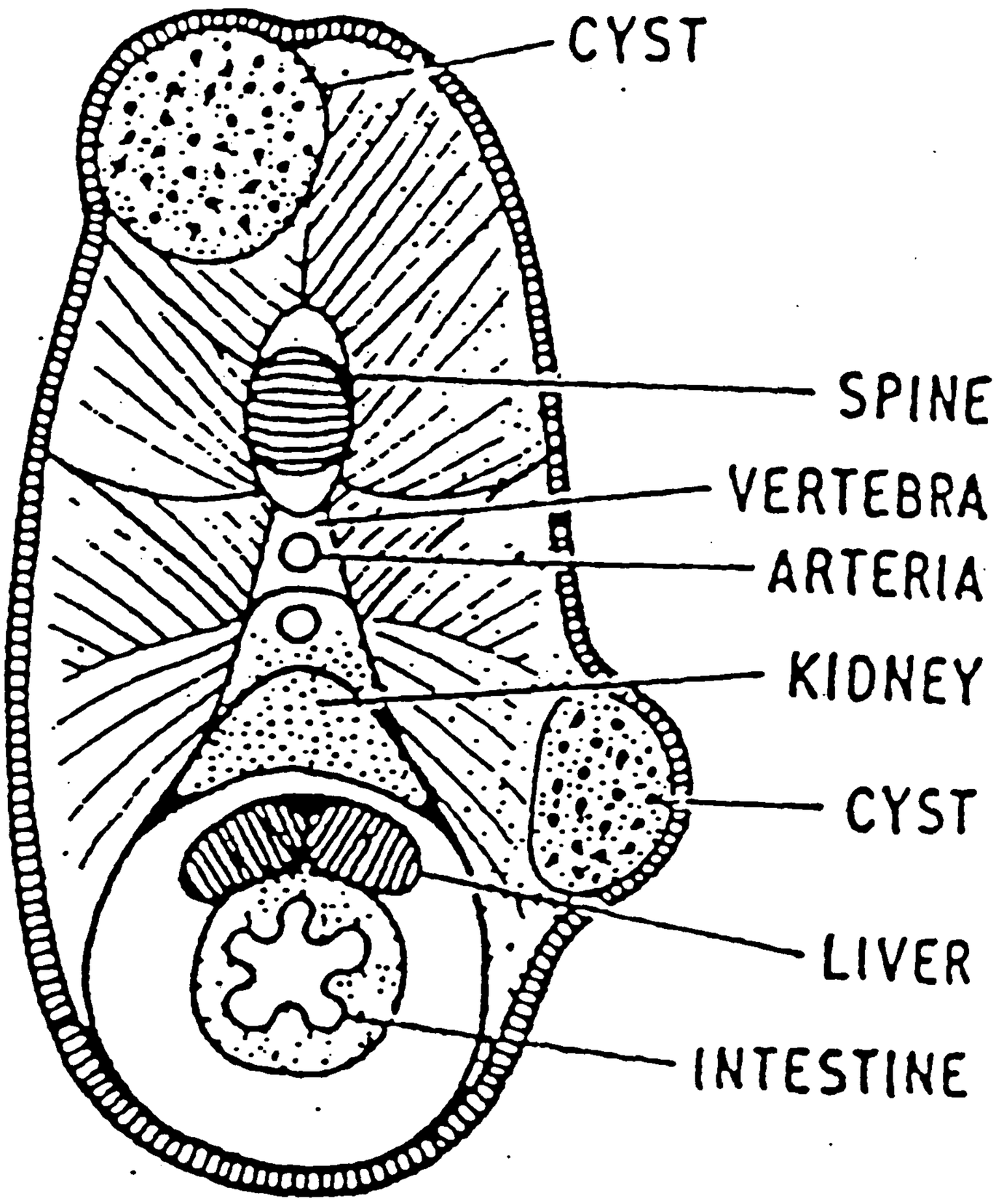


Figure 3.81: A transverse section through a stickleback (*G. aculeatus*) showing the position of cysts of the sporozoan, *Glugea anomala*, in the trunk musculature of the infected fish (Van Duijn, 1973).

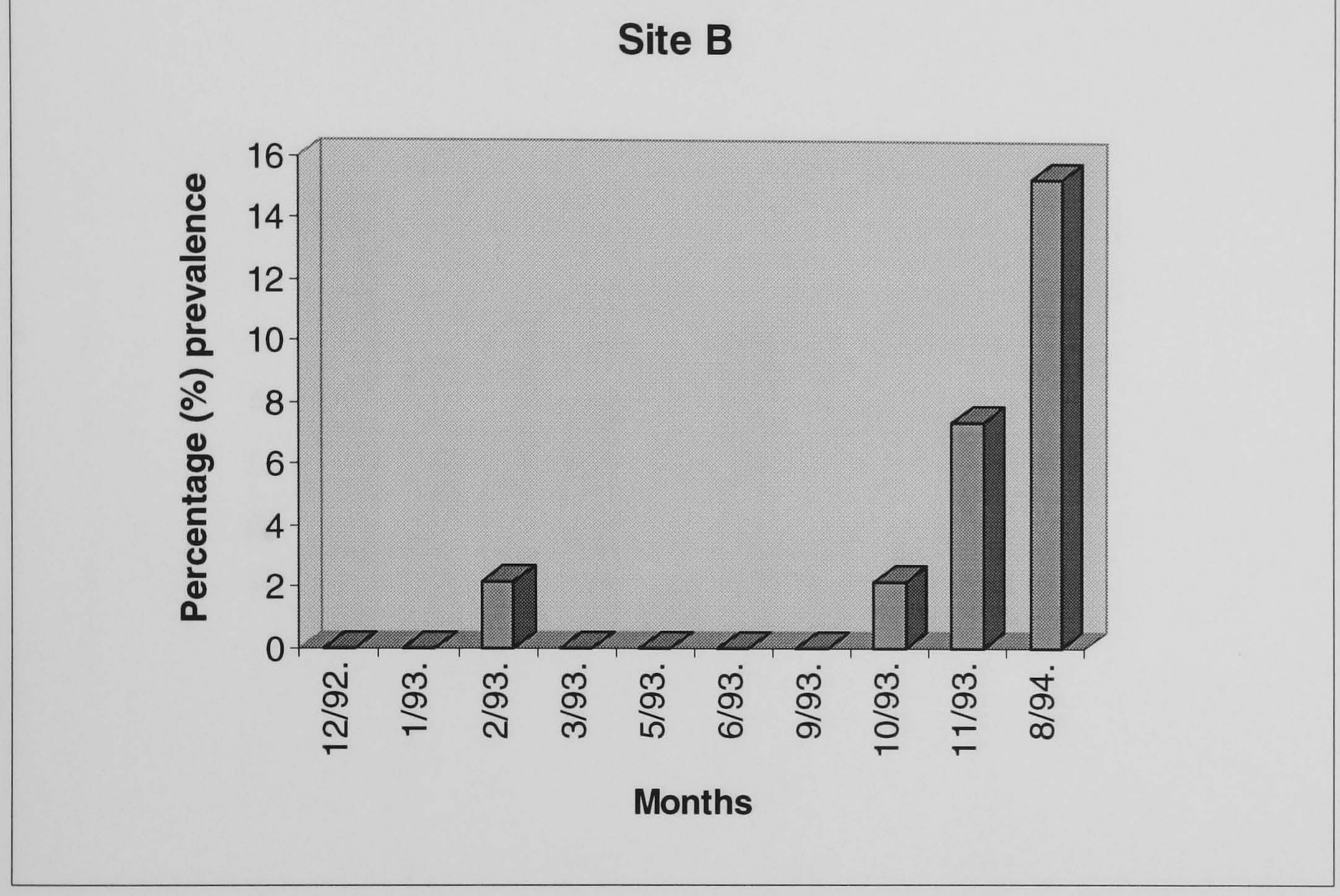


Figure 3.82: Prevalence of *Glugea anomala* infection in *G. aculeatus* at site B.

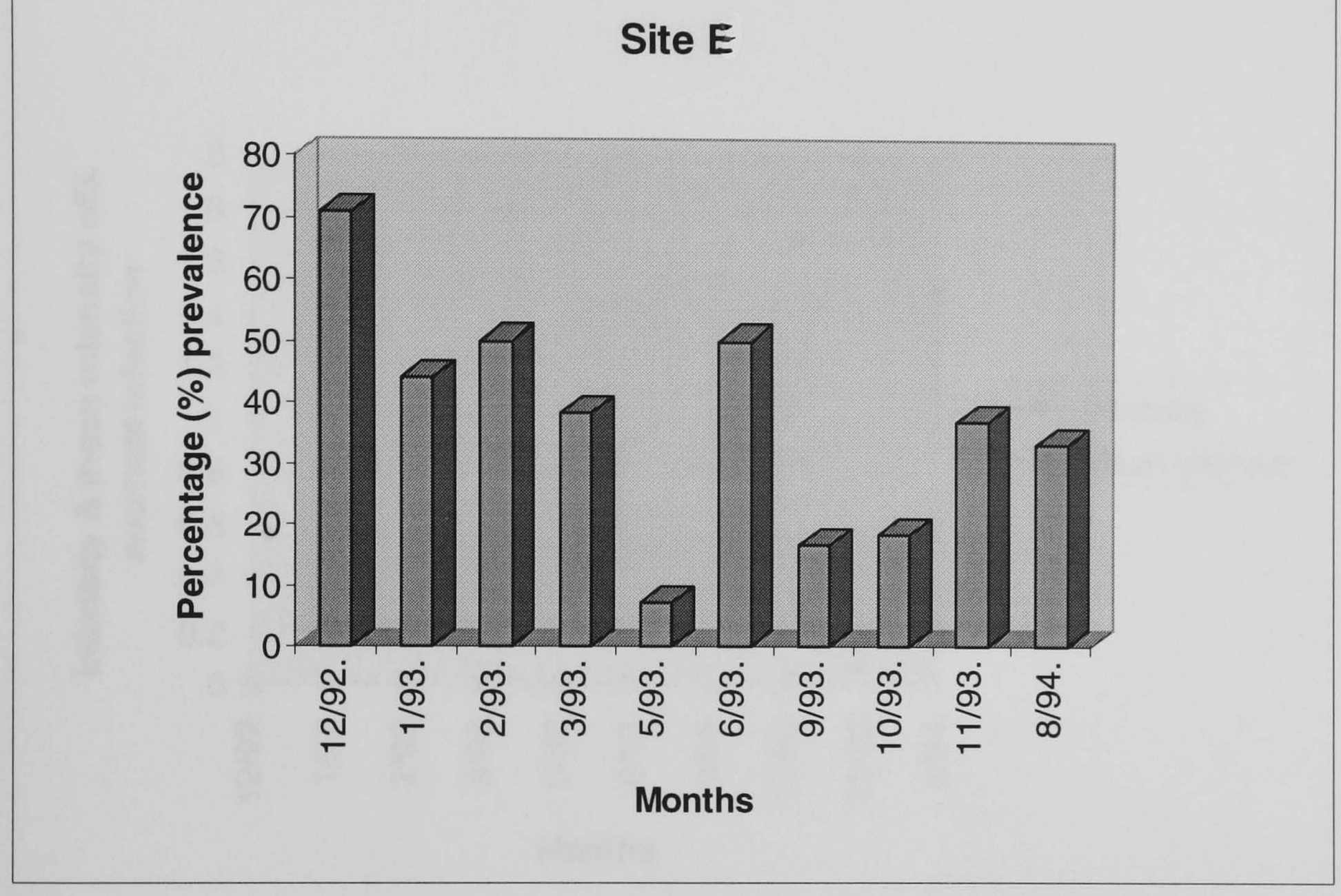


Figure 3.83: Prevalence of *Glugea anomala* infection in *G. aculeatus* at site E.

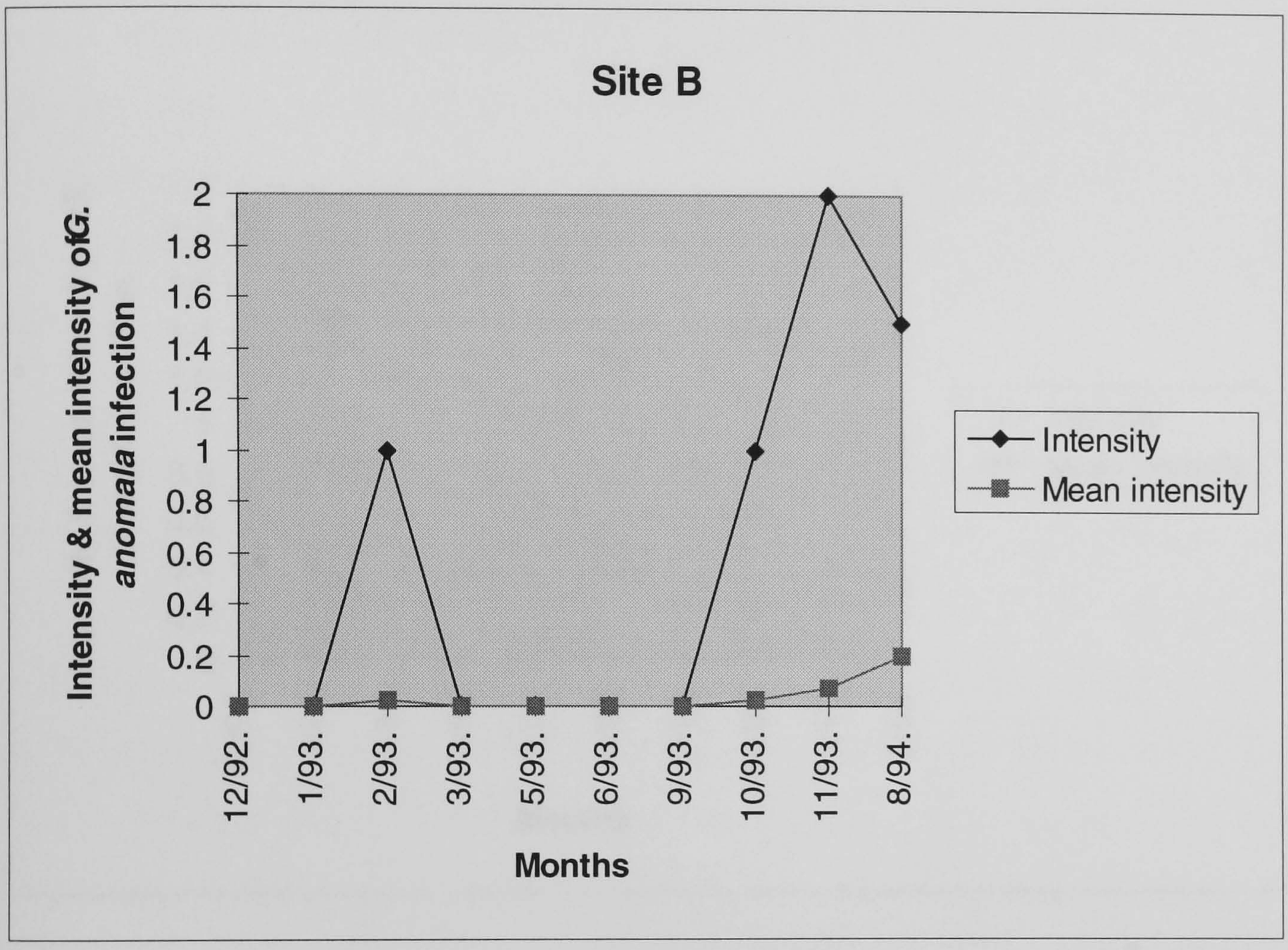


Figure 3.84: Intensity and mean intensity of *Glugea anomala* infection in *G. aculeatus* at site B.

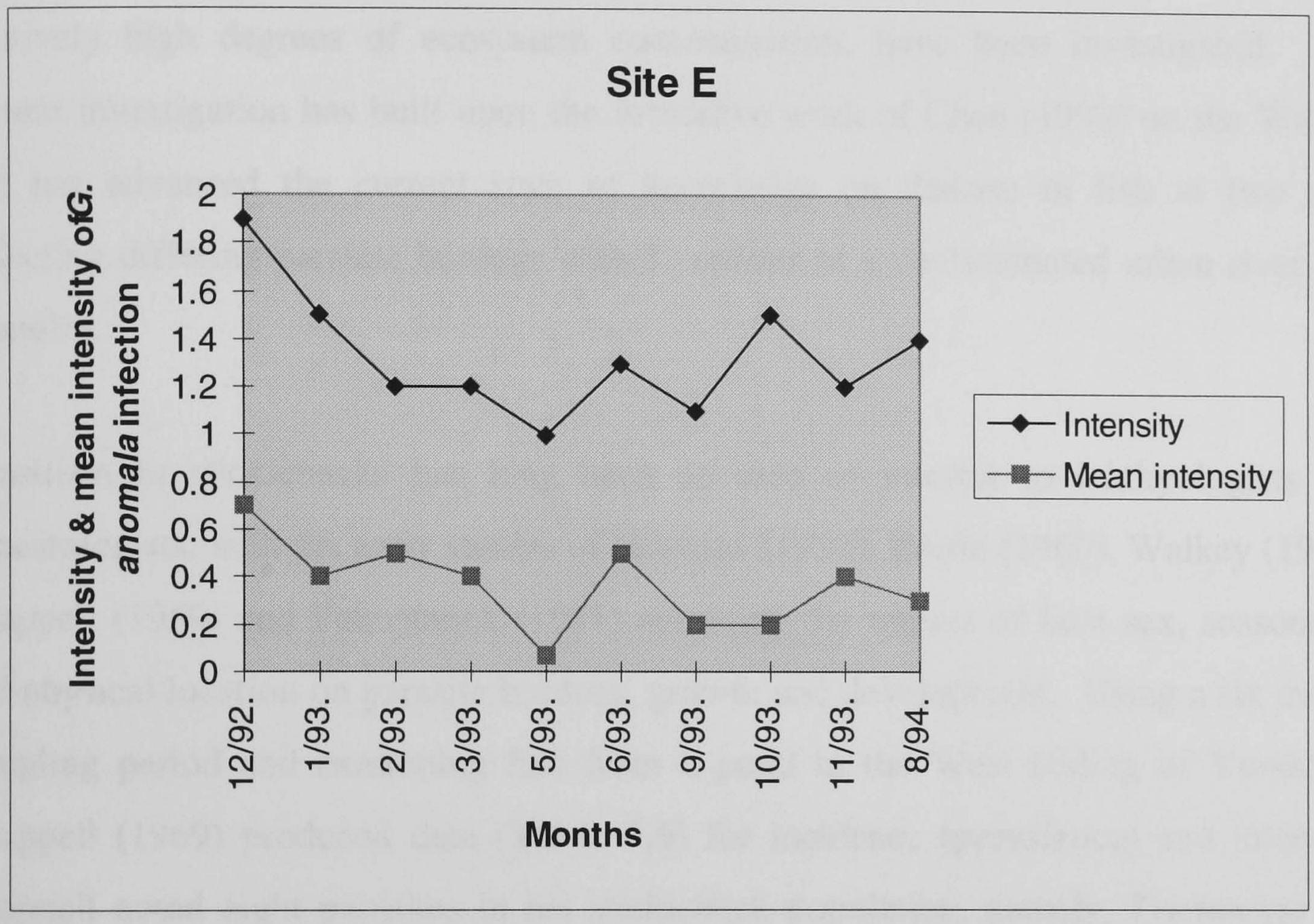


Figure 3.85: Intensity and mean intensity of *Glugea anomala* infection in *G. aculeatus* at site E.

3.4. Discussion.

There have been several previous investigations of the ecology and parasitology of British and European stickleback populations and a convenient summary has been provided of these by Wootton (1976). A study of this available literature, does however, indicate that comparatively few urban sites, especially those exhibiting relatively high degrees of ecosystem contamination, have been investigated. The current investigation has built upon the formative work of Chen (1992) on the Wandle and has advanced the current state of knowledge on disease in fish at two sites reflecting differing parasite burdens with *S. solidus* in a contaminated urban river (the Wandle).

Parasitism in sticklebacks has long been an area of interest to ichthyologists and parasitologists, with the early studies of Hopkins (1959), Berrie (1960), Walkey (1967), Chappell (1969) and Pennycuick (1971) assessing the impact of host sex, seasonality and physical location on parasite burdens, growth and development. Using a six month sampling period and examining fish from a pond in the West Riding of Yorkshire, Chappell (1969) produced data (**Table 3.6**) for incidence (prevalence) and intensity. Chappell noted eight parasites in his stickleback population, namely, *Trichodina* sp., *Gyrodactylus rarus* (found usually on the fins and body skin), the adult digenean *Phyllodistomum folium* in the bladders of the fish, metacercariae of *Diplostomum gasterostei* and *Diplostomum spathaceum* in the stickleback eyes, adults and plerocercoids of *Proteocephalus filicollis* in the alimentary tract, plerocercoids of *Schistocephalus solidus* in the peritoneal cavity and the acanthocephalans *Neoechinorhynchus rutili* in the rectum and intestine.

There are obviously differences and similarities in the species composition of the parasite community in Chappell's study in rural Yorkshire and that described here from the Wandle. *Trichodina*, a species of *Gyrodactylus*, *S. solidus* and *Proteocephalus filicollis* are shared by both communities. The Yorkshire sample lacked *Glugea*, while the Wandle sample lacked *Diplostomum* and *Neoechinorhynchus*. Considering the

shared parasite species the patterns of infection with *Trichodina*, *Gyrodactylus* and *Proteocephalus* were quite similar at the two distant and dissimilar locations.

Table 3.6: Incidence and intensity of parasitic infestation of *G. aculeatus* in a Yorkshire Pool (Chappell, 1969).

Percentage of fish infested (% incidence)						
Parasite	**Sept**	**Nov**	**Jan**	**Mar**	**May/June**	**Aug**
Trichodina sp	100	100	100	100	100	100
Gyrodactylus rarus	93	79	99	100	82	0.8
Phyllodistomum folium	34	49	48	48	45	55
Diplostomum gasterostei	97	99	97	88	95	41
Diplostomum spathaceum	97	98	97	95	92	98
Proteocephalus filicollis	40	41	35	28	41	12
adults	18	20	16	15	23	5
immature	26	26	28	22	29	6
Schistocephalus solidus	1	31	15	18	55	16
Neoechinorhynchus rutili ?	7	11	22	13	4	7
Number of fish in sample	100	100	91	99	91	120
Mean number of worms per infested fish (intensity of infestation)						
Gyrodactylus rarus	6.7	6.1	11.5	23.4	6.2	2.0
Phyllodistomum folium	1.8	1.8	1.9	1.9	2.1	3.1
Diplostomum gasterostei	4.5	4.9	3.8	3.3	4.7	5.6
Diplostomum spathaceum	5.9	4.7	4.9	4.5	4.0	12.6
Proteocephalus filicollis	1.5	1.5	2.4	2.4	4.5	1.3
adults	1.4	1.3	1.6	1.8	4.2	1.3
immature	1.3	1.4	1.8	1.7	3.0	1.1
Schistocephalus solidus	1.0	1.4	1.2	1.1	1.6	1.1
Neoechinorhynchus rutili ?	1.6	1.1	1.4	1.9	1.5	1.0

However, the infestation by the pseudophyllidean plerocercoids of *S. solidus* in Chappell's study was very different to that seen in this investigation. Typically, at site B the prevalence of the plerocercoids was almost nearly 100% during the winter months, declining to approximately 50% of this value during the summer sampling period. Chappell's data was the opposite to this with a lower prevalence during the winter and a higher prevalence during the summer for *S. solidus*. When the *S. solidus* intensities in Yorkshire and the Wandle are compared it is clear that the range 1 - 1.6 in Yorkshire is more similar to the range at site E in the Wandle (1 - 2.1) than it is to the range of values at site B (1.3 - 4.7) denoting much higher levels of infection. This is an

interesting difference considering that Chappell's site was rural and relatively uncontaminated while sites B and E in this study were urban, with high levels of inorganic contaminant input and heavy metals (Chen, 1992) present in the aqueous environment, diet and sediment.

Pennycuick (1971), again focusing attention upon a rural sampling site, Priddy pool in the Mendip Hills, reported a relatively high prevalence of *S. solidus* infection (88%). In common with Chappell, the prevalence of infection was found to be most intense in the summer with fewer fish having been recorded with infection during the winter. The Mendip sticklebacks were also infected with *Diplostomum gasterostei* and acanthocephalan *Echinorhynchus clavula* (?) **Table 3.7**. Additionally, these two studies, Chappell (1969) and Pennycuick (1971) have reported that the prevalence and intensity of parasitism throughout successive sampling periods is not subject to great seasonal or annual variability.

Table 3.7: Prevalence of infestation and mean number of parasite per stickleback in a Priddy Pool Mendip Hills Pennycuick (1971).

Parasite	% of infected fish	Mean no. of parasite / fish
S. solidus	88.3	4.40
D. gasterostei	56.1	9.35
E. clavula (?)	31.5	1.34

The detailed comparisons made above between the pattern of parasitisation of sticklebacks at two different, relatively unpolluted rural locations (Chappell, 1969; Pennycuick, 1971) and that found in the urban, relatively polluted River Wandle (this study) reveal a complex pattern of parallels and differences. The complexity of these relationships demonstrate very clearly that it will be very difficult to draw any simplistic conclusions about links between types and levels of parasitisation and levels of environmental contamination (see section 1.3.1 chapter 1).

Some tentative generalisations can, however, be made. Firstly, it is possible that the reduced parasite species diversity found in the Wandle (5 species) compared with that

in Yorkshire (8 species) is due to the presumed higher levels of contamination in the Wandle. It is conceivable, for instance, that the absence of *Diplostomum* sp, *Phyllodistomum* and *Neoechinorhynchus* in the Wandle sticklebacks is due to the absence of necessary intermediate hosts for these parasites in the macroinvertebrate fauna of the Wandle. Alternatively, free - living larval stages of these parasites might be sensitive to specific pollutants. [The Mendips population (Pennycuick, 1971) does not fit into this pattern, with only 3 parasite species. It seems possible, however, that this study did not explicity search for protozoan parasites].

Secondly, even for a parasite with a multihost indirect life cycle like *Schistocephalus* (bird, copepod, fish) there is no explicit evidence that site contamination leads to reduced parasite transmission. Prevalences and intensities of infection with this parasite are higher at site B in the Wandle than they are in Yorkshire and the Mendips.
A careful comparison between the patterns of infection in sticklebacks at sites B and E in the Wandle is especially instructive. These two sites are only about 1.61km apart in the same river, yet they show some consistent differences (over the sampling period employed) in their degrees and type of parasitisation. Particularly in the case of *Schistocephalus* infections, the detailed analysis of parasite populations which has been possible facilitates analysis of the plausibility of different hypotheses which might explain the parasitisation differences. One general conclusion which can be drawn from, for instance, **Figure 3.59**, is that at neither site is condition factor apparently influenced by *S. solidus* infection despite the fact that mean parasite index levels sometimes reach as high as 44%.

Considering prevalences and mean intensities where relevant, what is the overall comparative pattern of infection at sites B and E with the five parasites present there?. For *Trichodina* and *Gyrodactylus* it is very difficult to differentiate the infection levels at the two sites. Transmission and parasite development success for these two direct life cycle parasites must be very similar at sites B and E. For *Glugea*, however, another direct life cycle protozoan like *Trichodina*, there are quite distinct differences in infection load, judged by prevalences. Infection levels are clearly higher at site E than site B. For the remaning two indirect life cycle cestodes, however, *Schistocephalus* and

Proteocephalus, the site differentiation is in the opposite direction, with higher prevalences and mean intensites at site B than at site E.

This comparative pattern, like the comparison between Wandle parasites and those in rural unpolluted sites, does not allow simple generalisations. If there are significant ecological and contamination differences between sites B and E, despite their proximity, these differences are not influencing all five parasite species in the same way. For one species, (*Glugea*) site E is optimal, for two (*Schistocephalus* and *Proteocephalus*) site B is optimal, while for the other two (*Trichodina* and *Gyrodactylus*) the sites are equally utilisable.

How can the marked differences in infection with *S. solidus* at sites B and E be explained?

One suggestion might be that the slightly larger mean size of sticklebacks at site B, could, in conjunction with a size - dependent increase in infection level, simply explain the higher number of parasites at site B. Detailed analysis of host size - specific mean intensities at sites B and E (see **Figure 3.29**) reveals that this cannot be an appropriate hypothesis. For all host size classes apart from 1 - 1.9cm, the mean intensities at site B is considerably higher than that at site E. Fish population structure is not determining parasitisation differences.

A second hypothesis is that the smaller biomass loads of parasites in fish at site E might be driven by a smaller input of infective stages into the river at this location in terms of infected, egg - containing bird faeces. If this were the only explanation and the sites were, in all other ways, equally conducive to parasite growth and development, the size ranges of plerocercoids should be similar in the two sites even if the total numbers of parasites were fewer at site E than site B.

Indeed, if there were density - dependent constraints on plerocercoid size in individual fish caused by competition for limited resources, the mean size of individual plerocercoids at site B, where densities are higher, should be smaller than those at site E, where densities are lower. In one of the more surprising findings of this study, Figures 3.52 - 3.57 inclusive show that the opposite relationship holds. There is indeed,

a density - dependent restriction on plerocercoid weight at both sites, but, at all densities, plerocercoids at site E are smaller than those at site B.

This important finding, which has emerged from what seems to be the only parasitological study in the literature which has monitored the population structure of *Schistocephalus* plerocercoids, has profound implications. It must mean that although only 1.61km apart, sites B and E in the Wandle differ in some important respect or respects that curtail the growth and development of *Schistocephalus* plerocercoids at site E or enhance them at site B (or both). This seems to be true even if there are (unproven) differences in the input of infective stages to the aquatic habitat at the two sites.

It is beyond the bounds of the present study to speculate in any detail about what these influences on *Schistocephalus* growth and development might be. The study of Chen (1992) revealed that much of the flow of the River Wandle is based on sewage effluent and she assumed that this was the source of the metal contamination in the river. Because of this and other smaller polluted inputs to the river, it is "chronically polluted and throughout most of its length according to the National Water Council Classification Scheme (1990) it is class 3 : poor" (Chen, 1992). This suggests that both sites B and E are considerably contaminated and Chen's data on copper and cadmium pollution at sites B and E shows this is true. If one is searching within Chen's pollutant data for a damaging influence on *Schistocephalus* at site E which contrasts with site B there are no clear - cut candidates. For both copper and cadmium, however, levels in river sediment at site E are higher than at site B (390µg/g Cu at site E, 325µg/g at site B), 12.9µg/g Cd at site E, 9.8µg/g at site B. The facts, though, that these differences are relatively small against the underlying high level of pollution and that copper and cadmium levels in *Schistocephalus* themselves are higher at site B than site E, suggest that these heavy metals are not the key influences on *Schistocephalus*. Further studies are required to identify the real influences.

Chapter Four

4.1. Toxicity and metabolism of phenol and pentachlorophenol in sticklebacks with and without the parasite *S. solidus*

Introduction

Pollutant exposures, as short - term pulses or prolonged continuous inputs of xenobiotics into the water environment, have been reported to increase susceptibility to parasitic disease (Vladimirov and Flerov, 1975; Mohan and Summerville, 1988; Poulin, 1992). More recent studies have reinforced these findings and reported increased incidences of ecto- and endo-parasite infections following exposures to a range of different contaminants including hydrocarbons (Lom and Laird, 1969; Lehtinen *et al*, 1984; Khan and Kiceniuk, 1988; Khan and Thulin, 1991; Koskivaara and Valtonen, 1992).

Earlier studies have assessed the effects of a range of pollutants on the physiology, ecology and biochemistry of the stickleback (Erichsen - Jones, 1935, 1938, 1939, 1947a and 1947b; Matthiessen and Brafield, 1977; Boyce and Yamada, 1977; Chen, 1992). These investigations were concerned with uptake, toxicity and accumulation of metals and organic compounds. However, the effects of parasitic diseases on toxicity and pollutant disposition have been considered in only a few cases (Pascoe and Cram, 1977; Chen, 1992). A review of the current literature has revealed a lack of knowledge on the effects of cestode infections, for example *S. solidus* and *T. nodulosus*, on the toxicity and metabolism of organic compounds in fishes.

For many years, metabolism of substances in fish was a contentious issue. It was demonstrated as early as 1940s that phenyl glucuronides could be found in small cyprinid fish bile (Goodnight, 1942), however, the experimental data of Maickel *et al* (1958) suggested that fish were incapable of biotransformation.

In the early 1960s more light was shed on the ability of fish to biotransform. Huang and Collins (1962) and Adamson (1967), in two different investigations involving several phenolics, principally, p - aminophenol, o - aminophenol, p - nitrophenol, and 2,4 - dinitrophenol, and several species of fish , chiefly, spurdog (*Squalus acanthias*) goosefish *(Lophilus americanus*) and crucian carp (*Carassius carassius*), showed that biliary conjugates - sulphate and glucuronide, were present in fish bile and were also excreted into the aqueous immersion medium.

During the early - 1980s, Layiwola *et al* (1981, 1983a and b), using both conventional and radio - thin - layer chromatography, isolated glucuronide and sulphate conjugates of o-, m- and p-cresol and 1 - naphthol from several species of freshwater fishes, including, bitterling (*Rhodeus sericeus amarus*) bream (*Abramis brama*) crucian carp (*Carassius carssius*) goldfish (*Carassius auratus*) gudgeon (*Gobio gobio*), guppy (*Poecilia reticulata*), minnow (*Phoxinus phoxinus*) , perch (*Perca fluviatilis*), rudd (*Scardinius reythrophthalmus*) and tench (*Tinca tinca*). Layiwola (1982) further isolated, separated and characterised the formation of glucuronide conjugates of pentachlorophenol (PCP), 2,4 - dinitrophenol and 2 - naphthol in the bile of freshwater fish species. More recently Layiwola (1988) and Furay (1994) have demonstrated separation of biliary chlorophenol and chlorobenzene conjugates from the liver of the benthic flatfish, the flounder (*Platichthys flesus*) using more sensitive High Performance Liquid Chromatography (hplc).

Lech and Bend (1980) utilizing inhibitors of metabolism showed that glucuronide conjugation in rainbow trout may be rapid enough to significantly affect the toxicity of certain chemicals. They investigated the effect of salicylamide (2 - hydroxybenzamide) which is a phase II inhibitor on the acute toxicity of 3 - trifluoromethyl - 4 - nitrophenol (TFM), a sea lamprey lampricide to fingerling rainbow trout. It was found that salicylamide, at an exposure concentration of 25mg/l, produced no observable effect on the fish but decreased the LC50 of the phenol to approximately one - third of that of fish exposed to the toxicant. It was also found that pre - treatment of the fish with salicylamide lowered the amount of TFM glucuronide in blood and bile, and at the same time the levels of unconjugated TFM

were elevated. Also investigations done by Glickman *et al* (1977) using the phase I inhibitor, piperonyl butoxide, and the phase II inhibitor, salicylamide, showed that these inhibitors not only increased the acute toxicity of pentachlorophenol, but influenced their disposition metabolism in fish.

Stehly and Hayton (1989) in an investigation of the metabolism of pentachlorophenol in rainbow trout found that salicylamide reduced the metabolism of PCP and this was accompanyed by an increase in the levels of parent compound in the fish tissues. They concluded that metabolism was an important factor affecting the toxicity of chlorophenols in fish. Other examples of similar investigations include, for fish (Lech, 1974; Glickman *et al*, 1977, 1982; Lech and Bend, 1980; Karara and Hayton, 1988) and invertebrates (Ankley *et al*, 1991).

Smith *et al* (1993), in a series of trials involving the white sucker (*Gatostomus commersoni*), have examined the consequences of experimentally induced liver lesions on the metabolism and biliary retention of glycoconjugates of the carcinogen 3,4 benzopyrene (benzo[a]pyrene). They have been able to determine that the main mode of biliary conjugation of 3,4 benzopyrene is via the glucuronide pathway and that fish exhibiting neoplastic liver lesions have a reduced ability to excrete the 3, 4 benzopyrenyl glucuronide conjugate which is characterised by the high levels of biliary retention and the onset of serious cholestatic disease.

to

A laboratory investigation was carried out ascertain the effect of parasitic infection by the cestode, *S. solidus,* on the toxicity of phenol and pentachlorophenol to sticklebacks. It was thought that if there was a difference in susceptibility to the toxins that this could be due to a difference in their metabolism between parasitised and non - parasitised fish. This was investigated by determining the effect of phase I and phase II inhibitors on the toxicity of the phenols in the two groups of fish. It was also possible that with no difference in susceptibility that there would still be measurable differences in the metabolism of these compounds. To this end it was decided to determine the production of phenyl glucuronides in the bile of the two groups of fish.

4.2. Materials and Methods

4.2.1. Toxicity of phenolic compounds to stickleback

As described in **section 2.6.1.** the toxicity of phenol and PCP to parasitised and non parasitised fish was determined by employing 8 to 16 fish per each exposure concentration with these tests being conducted as either duplicates or triplicates. In each test regime, a control and five arithmetically spaced exposure concentrations were used. All experiments were carried out at a temperature of 4°C.

4.2.2. The effect of salicylamide and piperonyl butoxide on the toxicity of phenol to parasitised and non - parasitised sticklebacks

In a separate group of experiments, involving the toxicity of phenol to parasitised and non-parasitised fish, the metabolic inhibitors salicylamide (2-hydroxybenzamide) and piperonyl butoxide were used. In these experiments the fish were pre-exposed, for a duration of 48h to salicylamide or piperonyl butoxide at a concentration that resulted in no mortality. In this instance, groups of fish were exposed to separate concentrations of salicylamide (20mg/l) and piperonyl butoxide (5mg/l) for 48h prior to being immersed in the experimental concentrations of phenol (see appendix for details A 25-A 32).

4.2.3. Influence of metabolic inhibitors on the production of phenyl glucuronide in the bile

Groups of eight fish, comprising of separate batches of non - parasitised and parasitised fish, were exposed to a sub - lethal concentration for phenol 45.16μM and for PCP 0.37μM. These sub - lethal concentrations of phenols are 50% of the 96h LC_{50} concentration values.

Each group of fish was immersed in 10L of the aqueous media and each experiment was repeated three times. All fish were rinsed in tap water, and killed by a blow to the

head followed by decapitation. The fish were dissected and bile was collected in glassvials after puncturing the gall bladder. It was found necessary to pool together the bile of all 8 fish to obtain a sufficient volume for analysis. Control trials involving fish swimming in 10L of freshwater were also conducted.

4.2.4. Separation and analysis of samples

High performance liquid chromatography (hplc) was used to separate the parent compounds and their metabolites in the samples. The details of these procedures and identification of metabolites have been presented in **Chapter 2.**

4.2.5. Estimation of metabolites and parent compounds in the samples

In addition to separation and identification of the various compounds by hplc, an internal standard method was employed to quantify both the parent compound and their conjugates present in the bile. The estimation procedure has been described in **Chapter 2**. The internal standard used for the estimation of phenol sample was 2,3,5,6 - tetrachlorophenol and phenol was used for the estimation of pentachlorophenol. The choice of an internal standard is based on the retention time being different to that of the compounds under investigation.

4.3. Results

4.3.1. Toxicity of phenol to non-parasitised and parasitised fish

The fish in these experiments were divided into those with and without infestation by *S. solidus* and as described above this was determined from the swelling of their abdominal cavity. A further check was carried at the end of the experiment when the fish were disected. **Figure 4.1** and **Figure 4.2** show respectively the percentage of mortalities of non - parasitised and parasitised fish exposed to phenol during the 96hr exposure period. The **Figures 4.3** and **4.4** show the percentage mortalities in the two groups of fish exposed to phenol but with previous exposure to salicylamide and

Figures 4.5 and **4.6** show the mortalities of fish with the piperonyl butoxide treatment. (The toxicity data are recorded in the appendix).

The data indicate that the toxicity of phenol, to both the parasitised and non-parasitised sticklebacks, is the same. Even after 48h pre-exposure to salicylamide or piperonyl butoxide these figures were not found to change to any appreciable extent. In all cases the LC_{50} for phenol was 8.5 or 8.6 mg/l (90.32μM or 91.38μM).

The results for the effect of salicylamides are difficult to explain. Previous work has shown that the phase II inhibitor significantly *increased* the toxicity of phenol in fish (Lech and Bend, 1980; Layiwola 1982 and 1988; Furay, 1994). However, there is no previously reported work on the effect of salicylamide on the metabolism of phenol in sticklebacks and so caution is needed in interpreting these results.

The hypothesis on which this present chapter was based upon, was that in a diseased state the response of the fish to the toxic effect of the phenolic compounds, would be affected significantly. The data here have demonstrated that infection by *S. solidus* has no observable effect on the toxicity of phenol to the stickleback.

4.3.2. Toxicity of pentachlorophenol (PCP) to parasitised and non-parasitised fish

The toxicity was determined for pentachlorophenol (PCP) to sticklebacks, both parasitised and non-parasitised and the percentage mortalities are given in **Figure 4.7** and **Figure 4.8**. The LC_{50} values was in each case estimated to be 0.2mg/l and 0.75μM (The toxicity data are recorded in the appendix).

The 96h LC_{50} data also show that the toxicity of PCP, as reported for phenol, does not change in relation to whether or not the fish are infected or not infected with the cestode parasite. Further studies using metabolic inhibitors were not carried out due to this similarity and were not of interest to pursue. It can be said that both results for PCP and phenol, are somewhat pointers to interesting studies since they both indicate

that cestode parasitism may have no significant influence on mediating changes in the toxicity and toxic effects of these two phenolic compounds.

4.3.3. Bile biotransformed products of phenol and PCP in parasitised and non - parasitised fish

The chromatograms (**Figures 4.9 and 4.10**) show the peaks and retention times of the products recovered in the bile after 96h exposure to phenol and pentachlorophenol respectively. It was observed that only one conjugate of phenol or pentachlorophenol was present in the bile. This conjugate is the glucuronide of each compound as confirmed by hydrolysis with the enzyme, β - glucuronidase. The phenyl glucuronide or the pentachlorophenyl glucuronide conjugate with retention time of 1.49min decreased in peak size after the 16h digestion with β - glucuronidase while the parent compounds, phenol with a retention time of 3.50min and pentachlorophenol with a retention time of 2.39min increased in peak size (**Figures 4.11** and **4.12**). The analytical result demonstrates that both parasitised and non - parasitised fish have the capacity to biotransform phenol and pentachlorophenol. This shows that the presence of parasite in the peritoneal cavity has no influence on the ability of the fish to biotransform phenol or PCP.

4.3.4. Quantification of biotransformed products and parent compounds

After the hplc separation and enzyme identification of the bile excretory products, it was† to determine if there was any influence of the parasite on the amount of the excreted products. This was investigated by using the internal standard method to verify the quantity of the glucuronide conjugates and their parent compounds.

Table 4.1 and **Table 4.2** show the peak height-ratio of the compounds to the internal standard method; and the amount calculated from the relationship (see **Chapter 2**). These appears to be no trend in these results, however, because only one sample was available it was not possible to undertaken any statistical tests. In the non-parasitised fish the amount of phenyl glucuronide was 18.0 μg/ml whereas that of parasitised fish

† necessary

was 24.0 μg/ml, and also the amount of parent compound was 25.5 μg/ml and 34.5 μg/ml respectively. If the increase in phenyl glucuronide indicated a greater degree of metabolism in parasitised fish it would have been expected that there would have been a reduction in the concentration of the parent compound in the bile.

In the non-parasitsed fish the amount of PCP glucuronide was 16.5 μg/ml and 18.0μg/ml in the parasitised fish. The concentration of the parent compound were 33.0 μg/ml in the non-parasitised and 27.0 μg/ml in the parasitised fish.

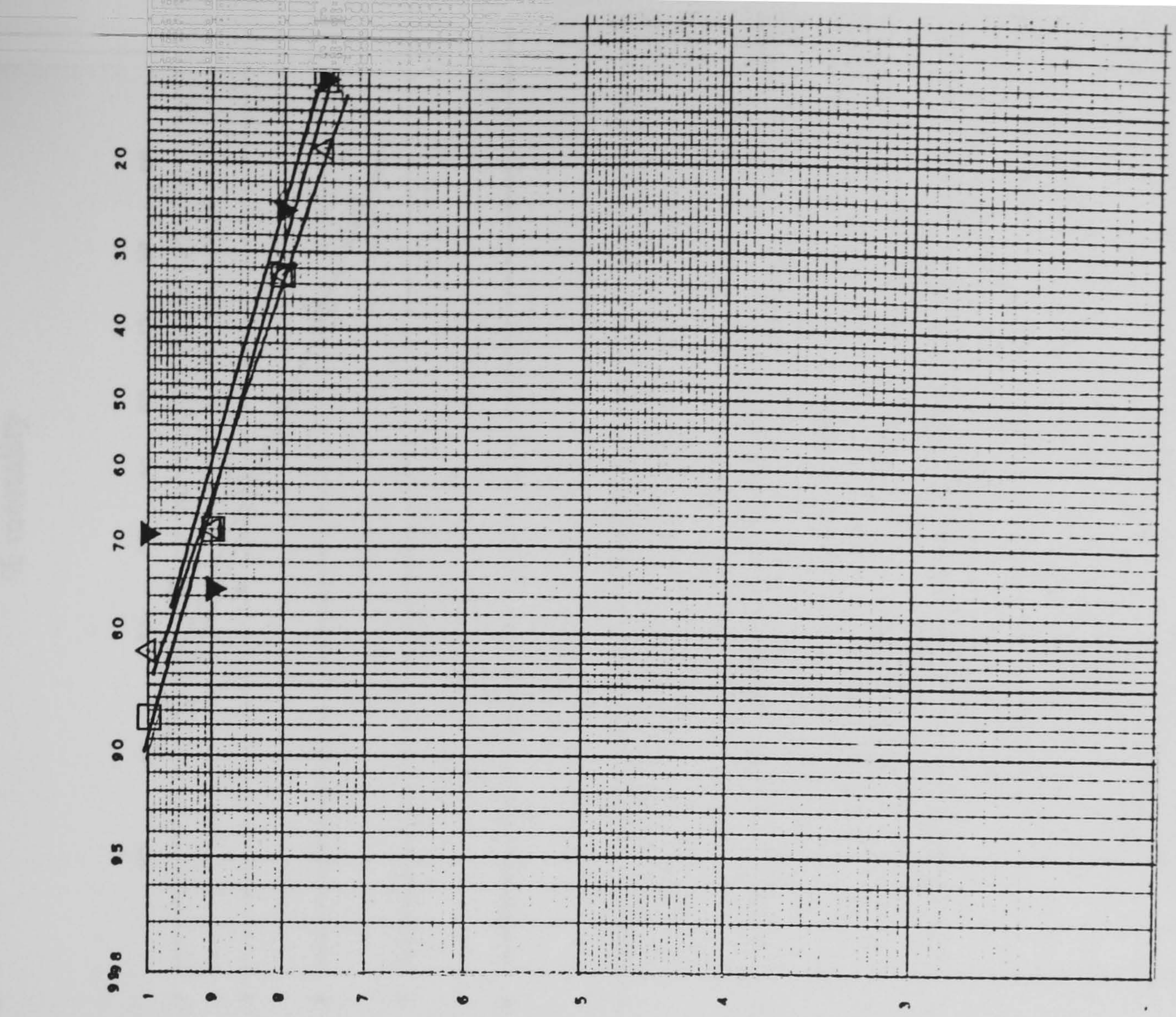


Figure 4.1: Percentage mortality of non - parasitised fish exposed to phenol for 96hr. (3 experiments)

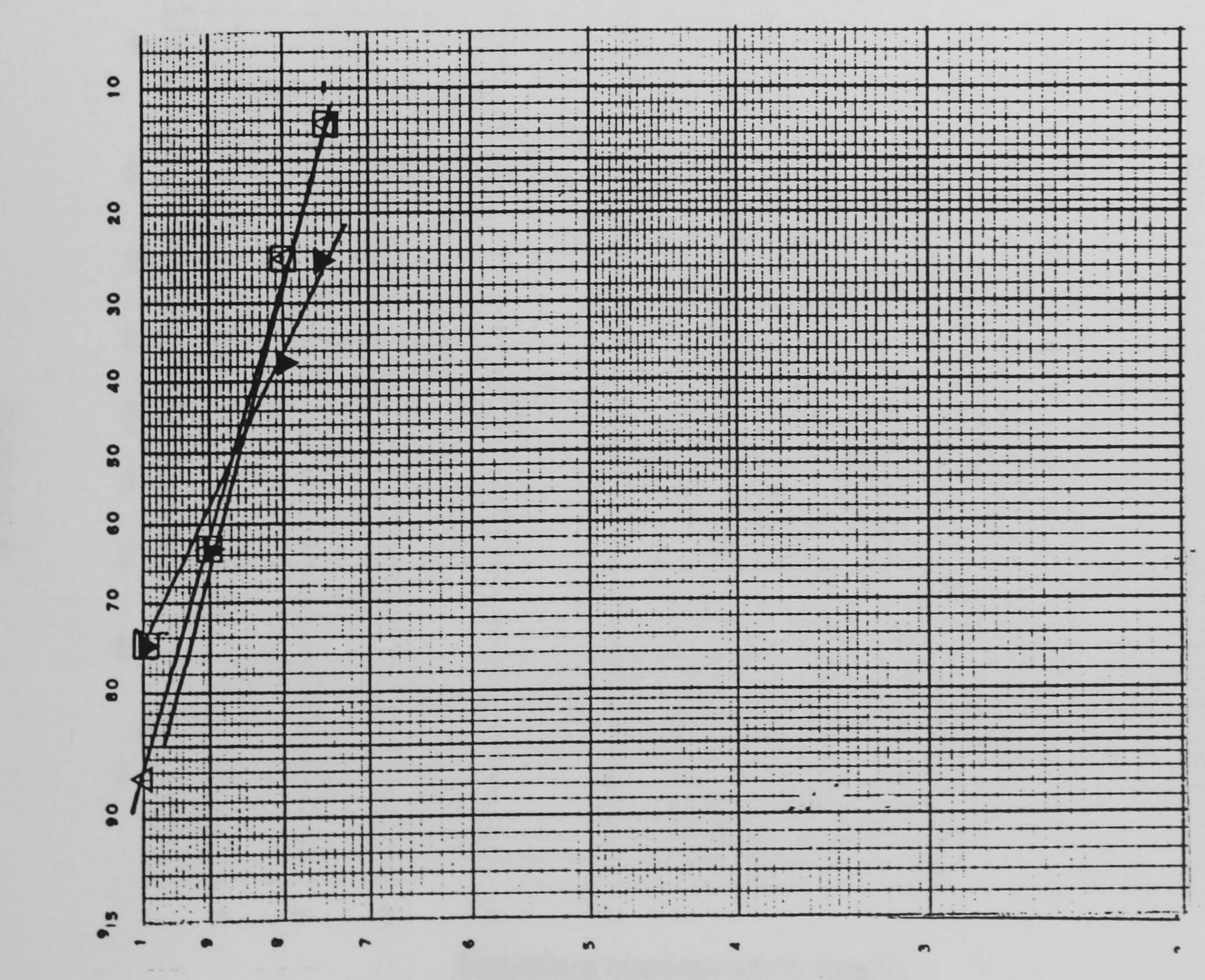


Figure 4.2: Percentage mortality of parasitised fish exposed to phenol for 96hr. (3 experiments).

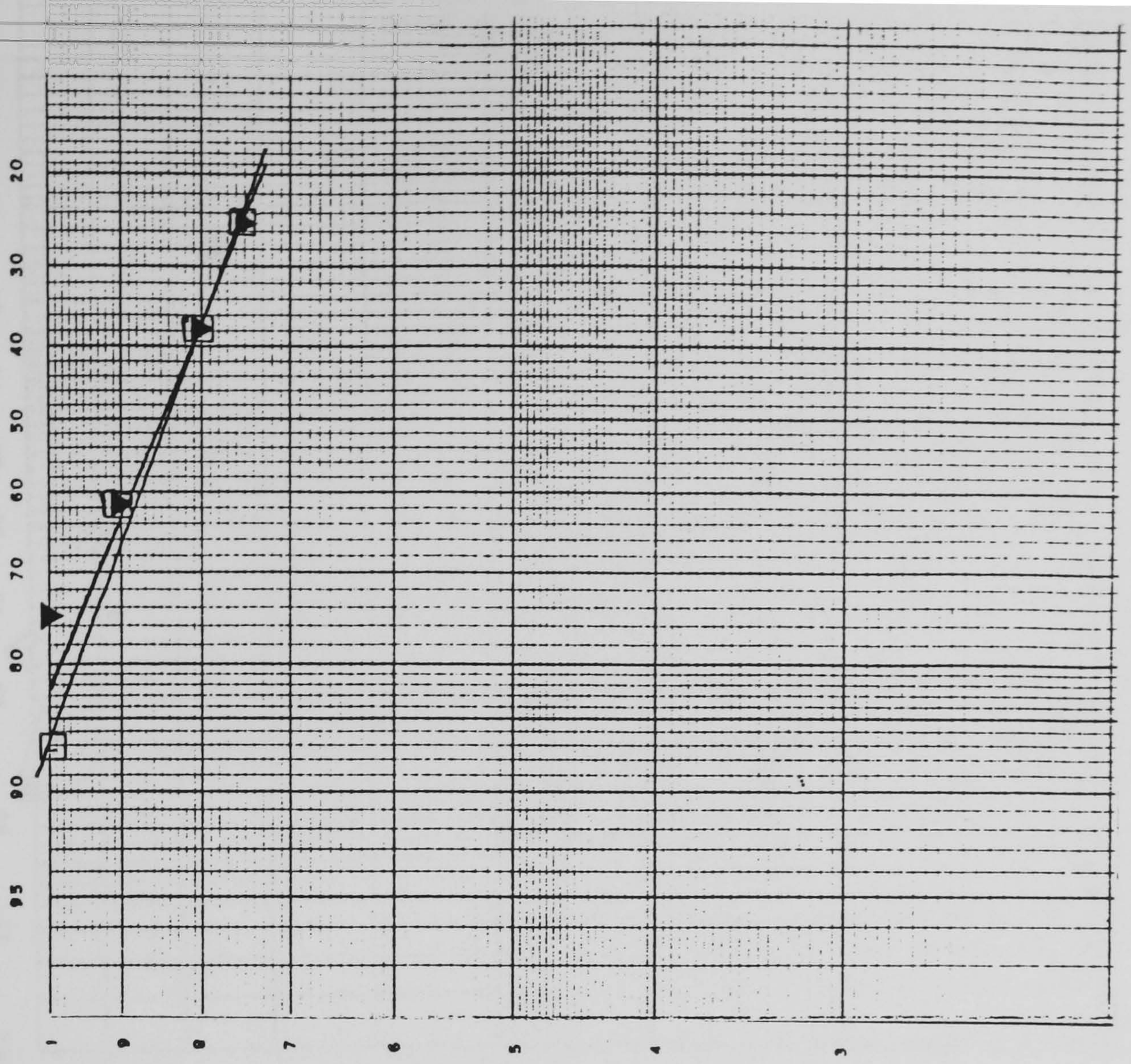


Figure 4.3: Percentage mortality of non - parasitised fish exposed to phenol pre - exposed to salicylamide for 96hr. (2 experiments).

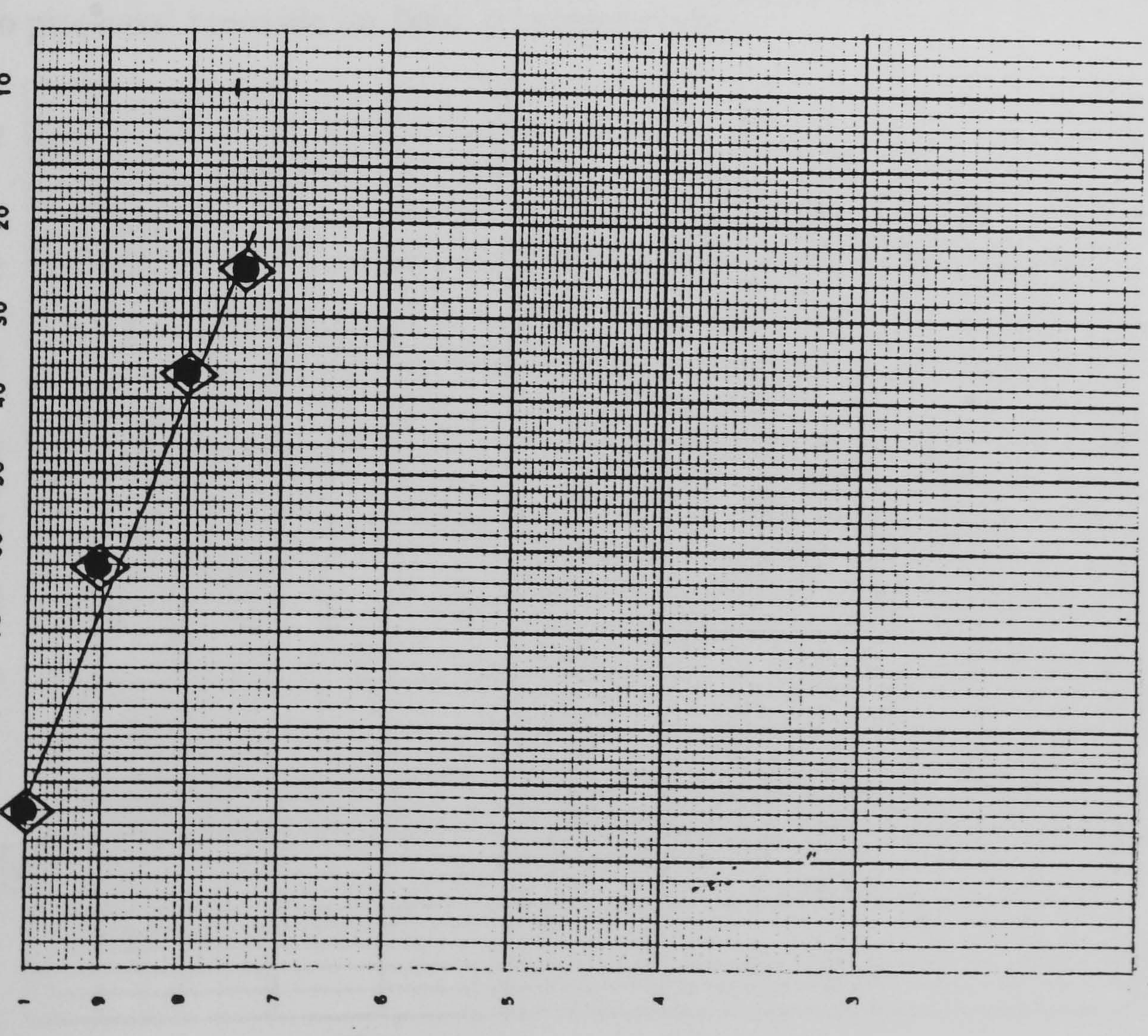


Figure 4.4: Percentage mortality of parasitised fish exposed to phenol pre - exposed to salicylamide for 96hr. (2 experiments).

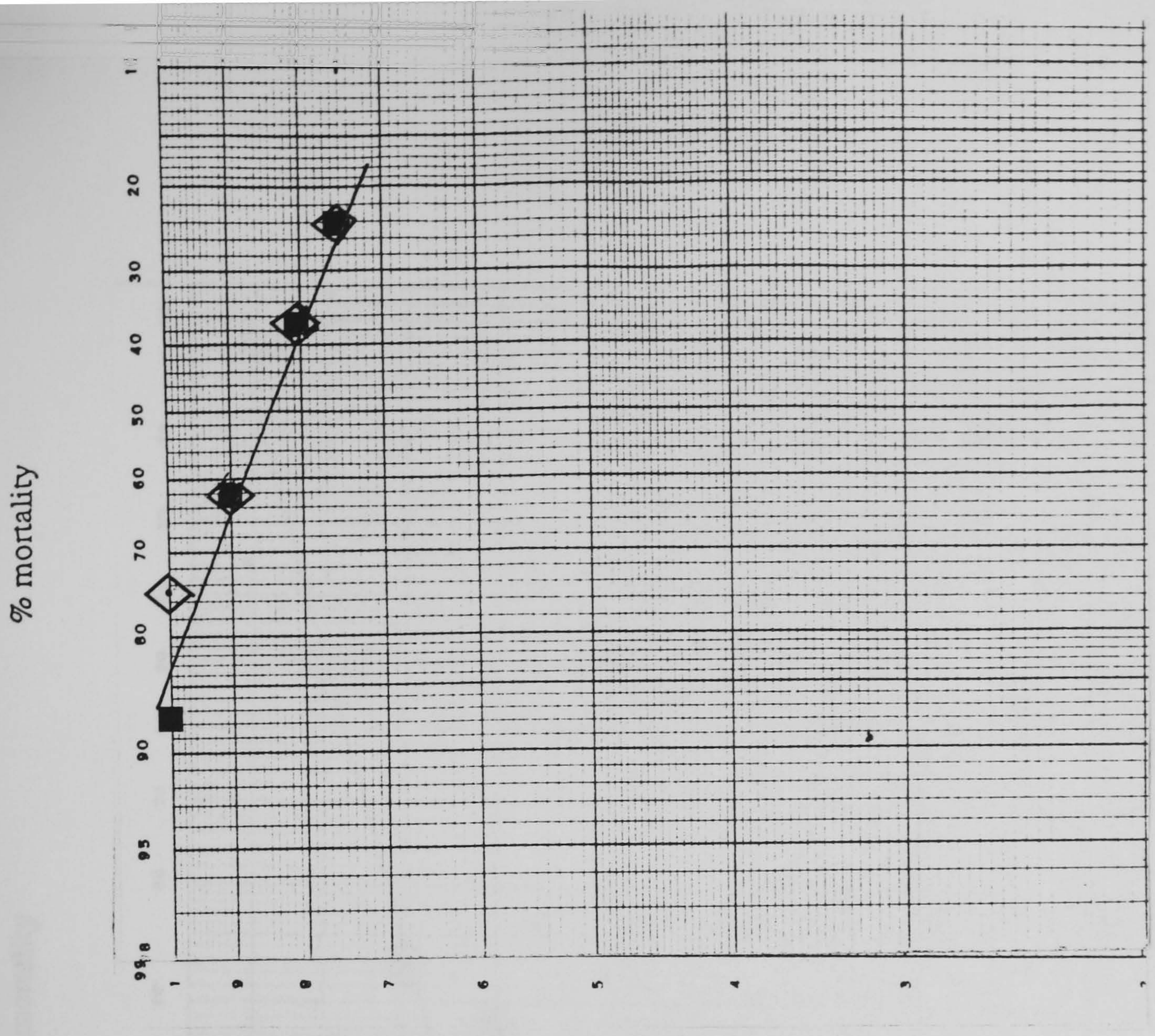


Figure 4.5: Percentage mortality of non - parasitised fish exposed to phenol pre - exposed to piperonyl butoxide for 96hr. (2 experiments).

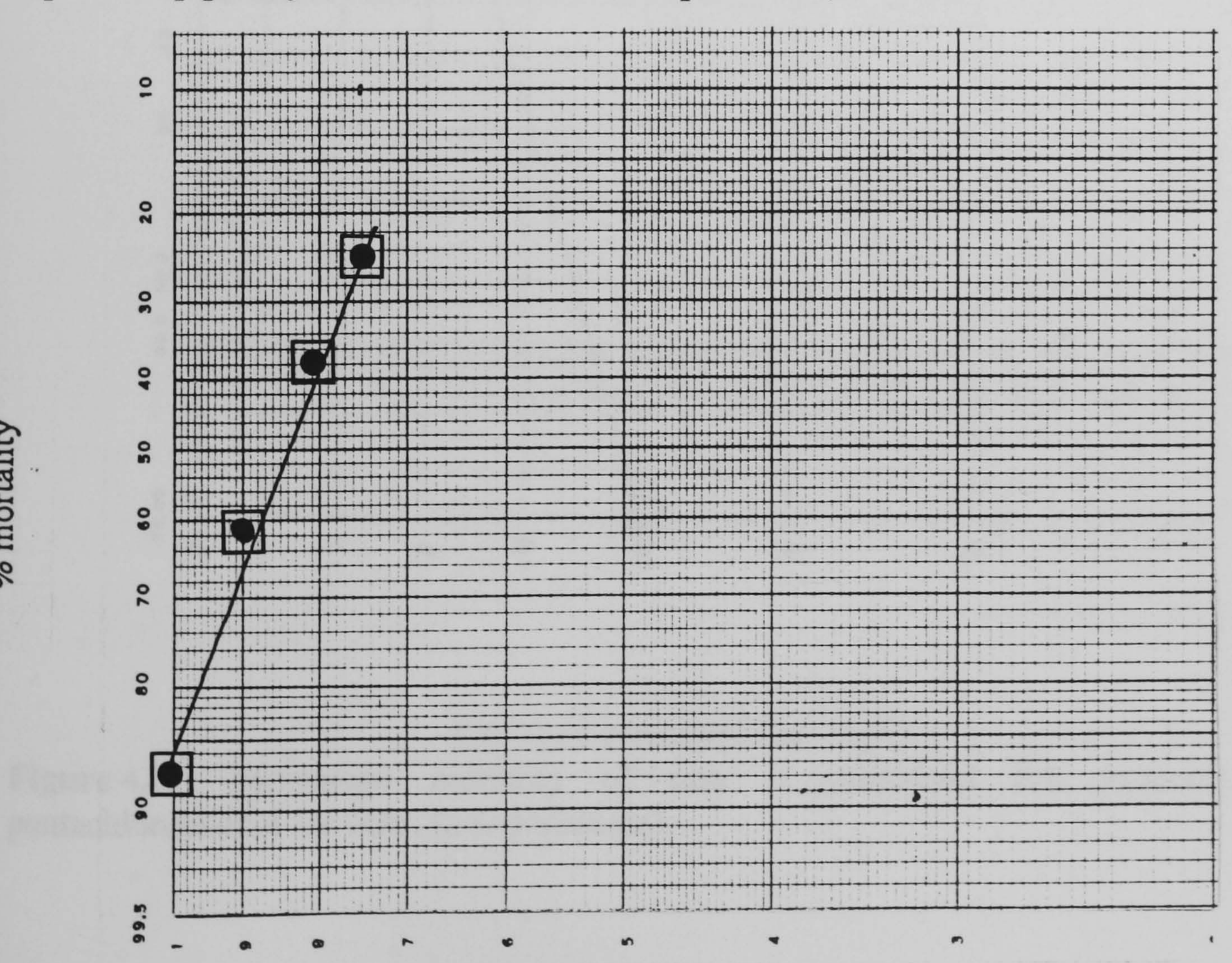


Figure 4.6: Percentage mortality of parasitised fish exposed to phenol pre - exposed to piperonyl butoxide for 96hr. (2 experiments).

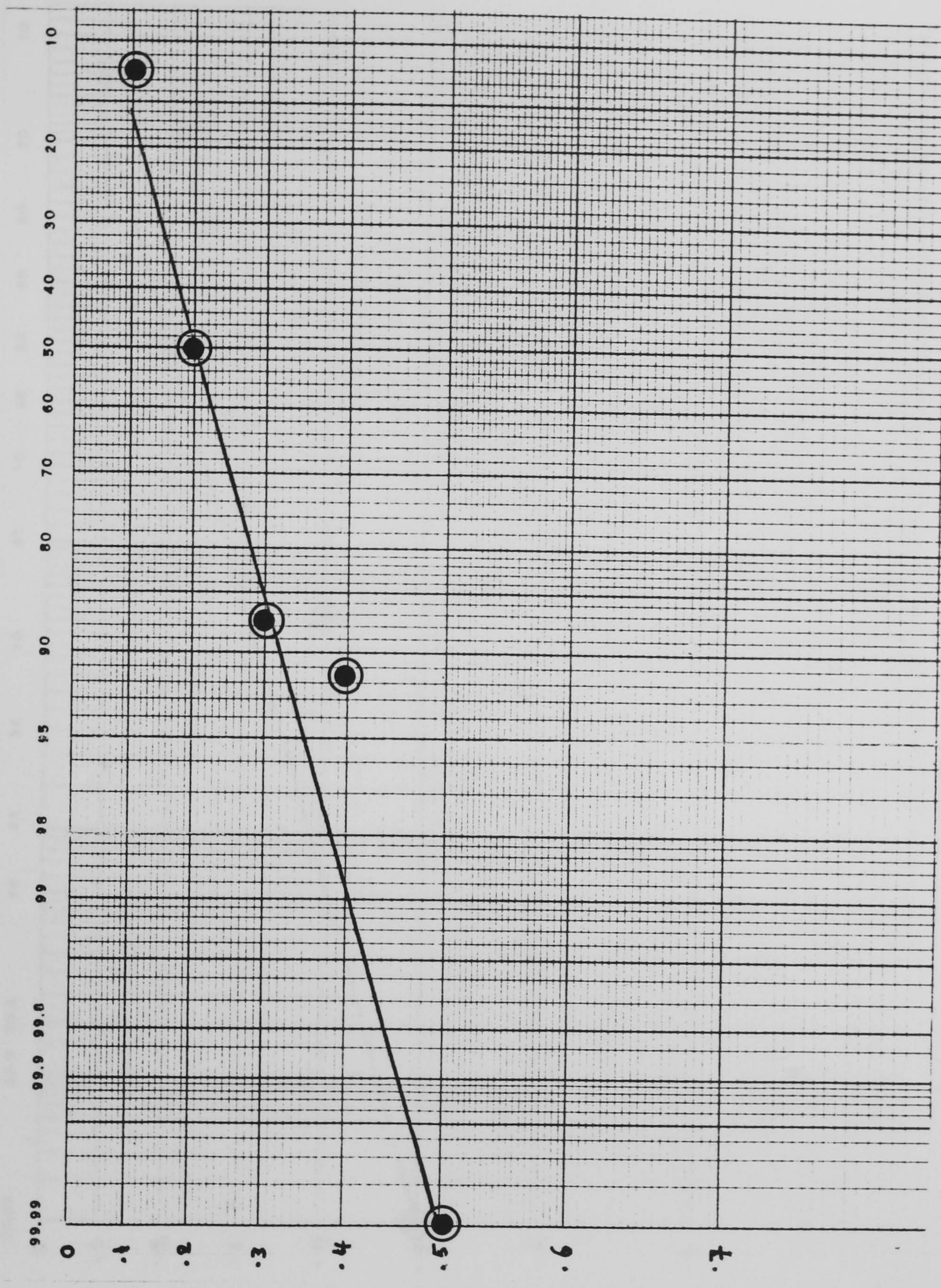


Figure 4.7: Percentage mortality of non - parasitised fish exposed to pentachlorophenol for 96hr. (2 experiments).

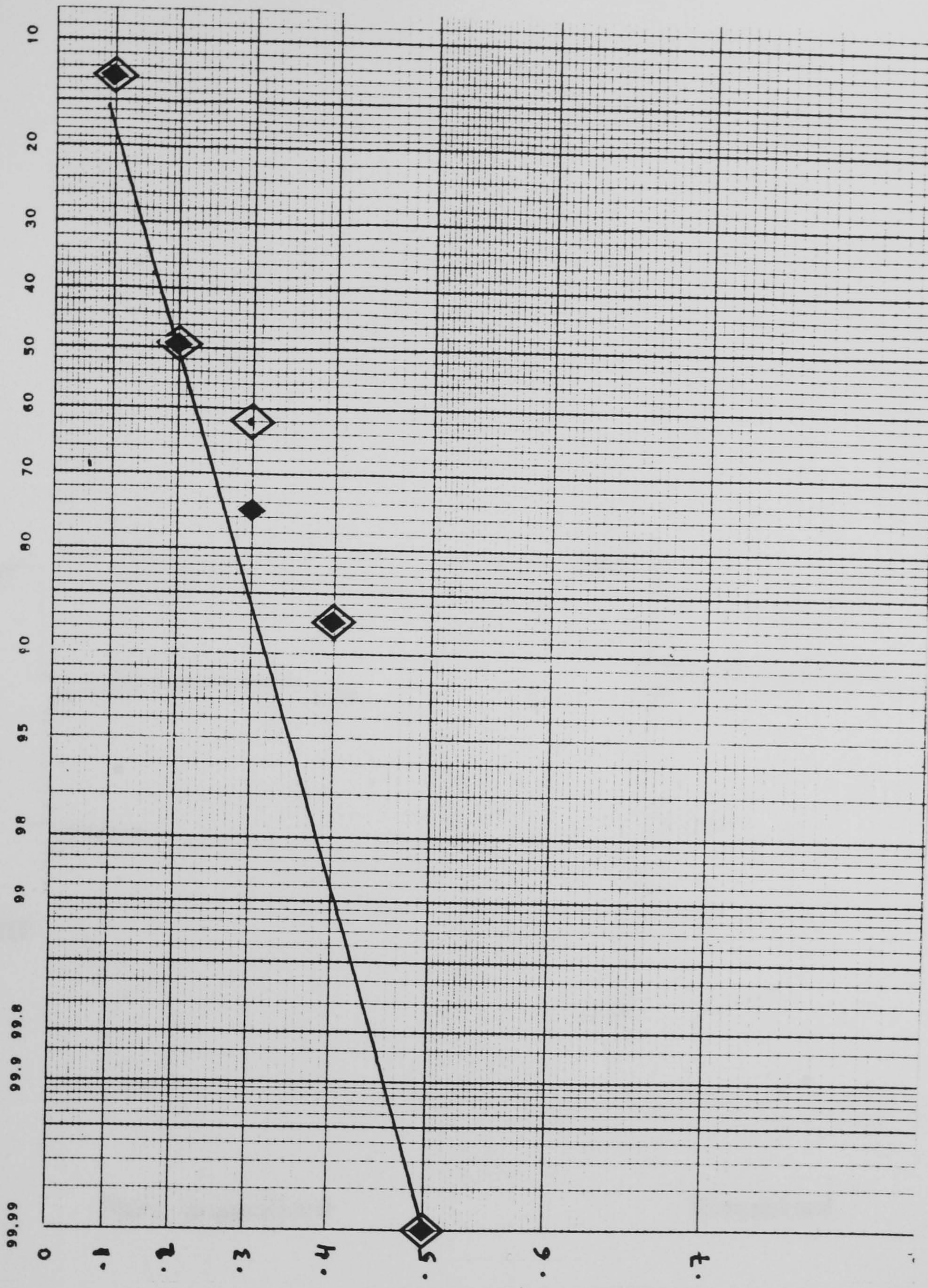
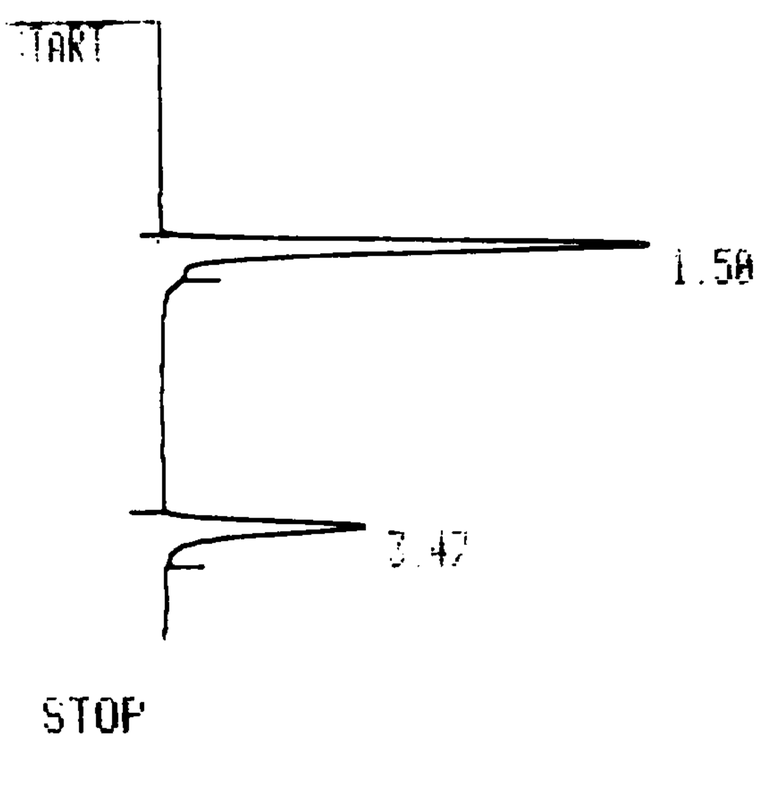
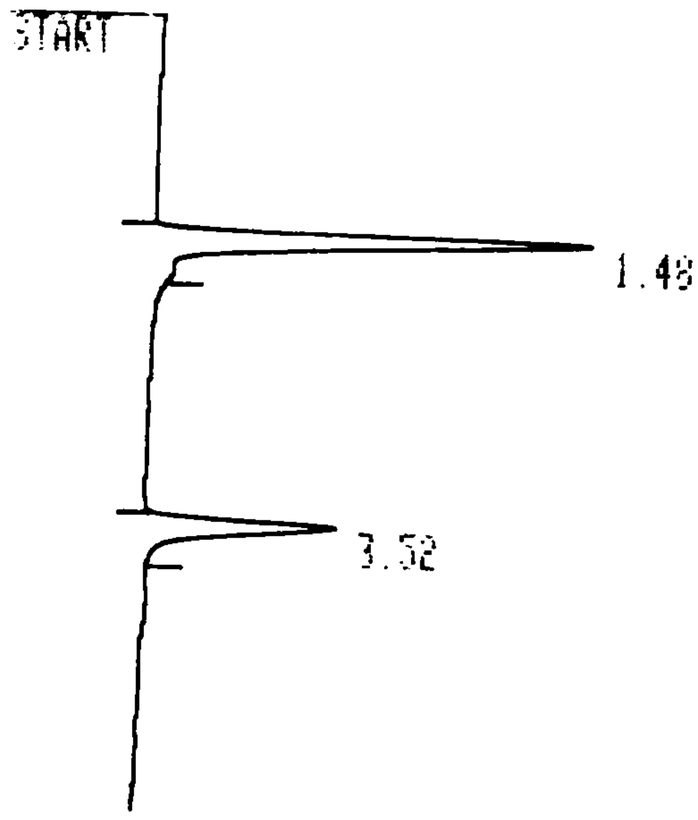


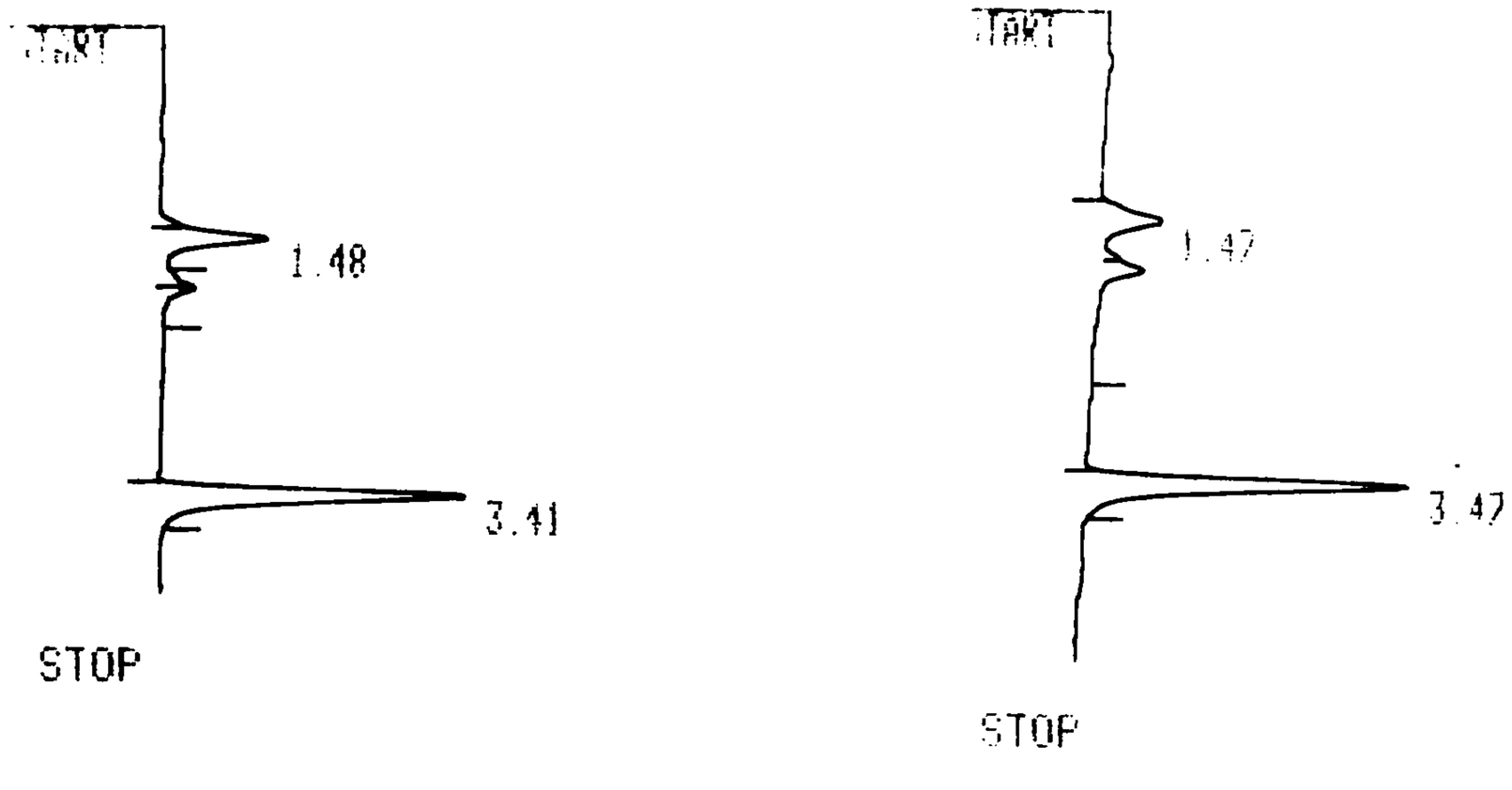
Figure 4.8: Percentage mortality of parasitised fish exposed to pentachlorophenol for 96hr. (2 experiments).





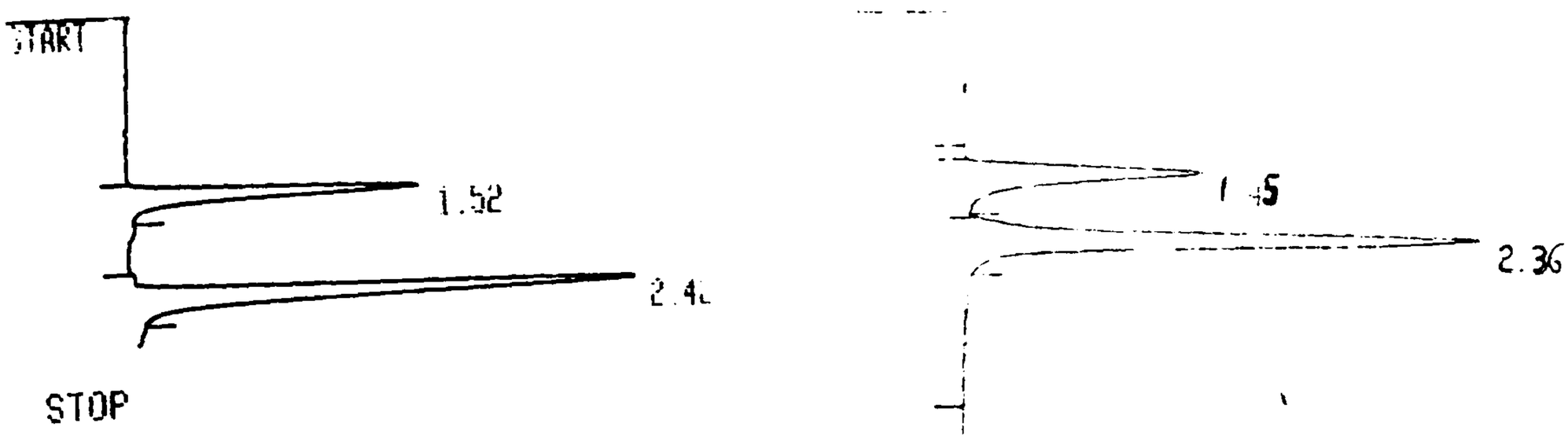
Non - parasitised **Parasitised**

Figure 4.9: Hplc chromatograms showing the peaks and retention times of phenol and its conjugate recovered in the bile of non - parasitised and parasitised fish after 96h exposure preiod.



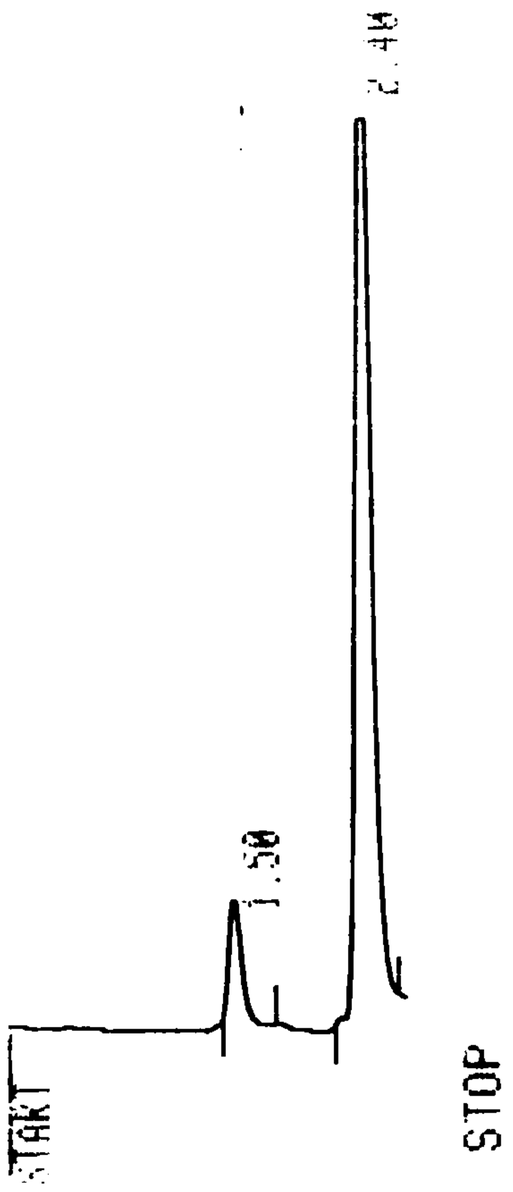
Non - parasitised **Parasitised**

Figure 4.10: Hplc chromatograms showing the peaks and retention times of PCP and its conjugate recovered in the bile of non - parasitised and parasitised fish after 96h exposure preiod.

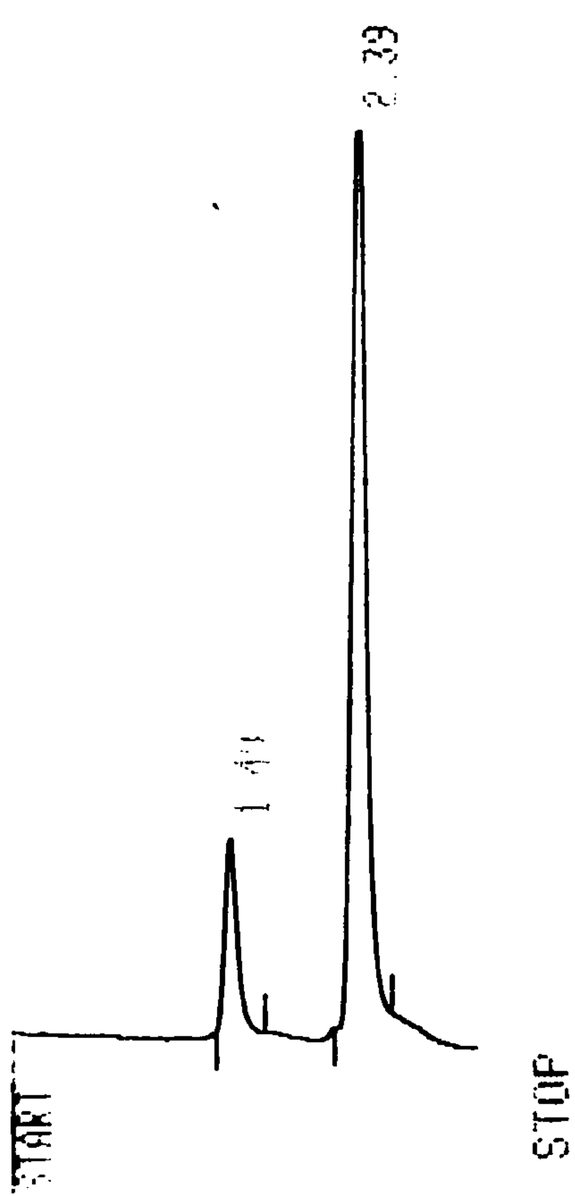


Non - parasitised **Parasitised**

Figure 4.11: Hplc chromatograms showing the enzyme hydrolysis of phenyl glucuronide and the increase in the peak of phenol in the bile of non - parasitised and parasitised fish.



Non - parasitised



Parasitised

Figure 4.12: Hplc chromatograms showing enzyme hydrolysis of pentachlorophenyl glucuronide and the increase in the peak of PCP in the bile of non - parasitised and parasitised fish.

Table 4.1: Concentration of phenol and its conjugate in the bile.

Compounds	Non - parasitised fish		Parasitised fish	
	Height Ratio	µg/ml	Height Ratio	µg/ml
Phenyl Glucuronide	1.2	18.0	1.6	24.0
Phenol	1.7	25.5	2.3	34.5

Table 4.2: Concentration of pentachlorophenol and its conjugate in the bile

Compounds	Non - parasitised fish		Parasitised fish	
	Height Ratio	µg/ml	Height Ratio	µg/ml
PCP glucuronide	1.1	16.5	1.2	18.0
PCP	2.2	33.0	1.8	27.0

4.4 Discussion

Toxicity of phenols has always been an area of great interest as numerous studies have reported the toxicity of chlorophenols to fishes. (Goodnight 1942; Kobayashi *et al,* 1969; Adelman *et al*, 1976; Adema and Vink, 1981; Saarikoski and Viluksela, 1982; Layiwola, 1982 and 1988; Shigeoka *et al*, 1989a and b; McCarty *et al*, 1991; Saito *et al*, 1991 and Smith *et al*, 1994).

More work has also attempted to understand the significance of specific metabolic pathways in the mediation of toxic effects especially with the use of metabolic inhibitors (Lech and Bend, 1980; Layiwola 1982 and 1988; Ankley *et al* 1991; Furay, 1994). Aspects of disease have also been incorporated into toxicological studies, for example, Smith *et al* (1993) investigated the relationship between cholestatic disorders and benzo[a]pyrene metabolism in white suckers (*Catostomus commersoni*).

Whilst there have been a number of studies on the effects of contaminant exposure on the susceptibility of fishes to parasitism, for example the work of Paperna (1975) on the infection by *Bebedenia* sp. in fishes following exposure to submarine oil, there have been relatively few investigations on the effects of parasitism on the acute toxicity of organic chemicals (Pascoe and Cram, 1977). The work that does exist on parasite - toxicity interaction, has been reviewed by McKenzie *et al* (1995).

Sticklebacks are generally infected by parasites such as *S. solidus*, *G. anomala*, *P. filicollis, T. reticulata* and *G. arcuatus*. *S. solidus* has been chosen for the study in this section to follow in line with earlier work in this thesis. This section set out with the hypothesis that cestode parasitism would have an effect on the acute toxicity of phenols to fish. However, this is not so as there was no different response recorded from the exposure of *S. solidus* infested stickleback to phenols. Even the employment of metabolic inhibitors to further elucidate the hypothesis was not helpful as indicated in the results. Whilst this is the obvious conclusion, it is also possible that infection with other parasites could have had an effect.

Although the hypothesis that *S. solidus* may affect phenolic acute toxicity did not hold, the toxicity data for phenol and PCP in sticklebacks in this work corresponds with that of published literature. For example the toxicity of PCP to stickleback is 0.75μM which is very similar to the values reported by Sloof (1983) for rainbow trout (*Salmo gairdneri* [*Oncorhynchus mykiss*]) 0.75μM, guppy (*Poecilia reticulata*), 3.19μM, Japanese medaka (*Oryzias latipes*) 4.13μM and fathead minnow (*Pimphales promelas*) 0.79μM. Values for flounder, variously reported by Layiwola (1988), and Smith *et al* (1994) yielded data that indicated that the toxicity of PCP to this benthic species was 2.90μM. These authors indicated the increased toxicity and sensitivity of these fish species to PCP as opposed to the base - member of the chlorophenol homologous series, phenol. These data are consistent with those reported here for sticklebacks where a very large increase was noted between the acute toxicity of phenol and PCP (90.31μM and 0.75μM respectively).

The results for the effect of piperonyl butoxide and salicylamide inhibitors were rather surprising, especially in the light of previously published results. It was expected that because the phenols would not be further hydroxylated by phase I enzymes that piperonyl butoxide would have no effect. However it was thought salicylamide would inhibit glucuronidation, resulting in large amounts of parent compound in the body which in turn would lead to in an increased toxicity. The fact that this did not occur suggests that perhaps the dose of salicylamide was too low.

The experiments in which the phenyl glucuronide was determined in bile indicated that there was no difference in the metabolism of phenols in the infected and non - infected fish. It is feasible that other metabolic pathways were involved but the hplc chromatograph indicated that glucuronide was the major one.

Schistocephalus solidus infects the peritoneal cavity of sticklebacks and as such it appears that it has no effect on the susceptibility of the fish to phenols. Although the literature does suggest that the parasite can have an effect on the general well - being of the fish (Walkey and Meakins, 1970; Giles, 1987; Milinski, 1984, 1990; Tierney et

al, 1996). The next section examines the effect of a specific liver parasite in perch (*Perca fluviatilis*) on the metabolism of phenol.

Chapter Five

Biochemical and histological investigations on perch *Perca fluviatilis* livers infected with the tapeworm, *Triaenophorus nodulosus*

Section I

5.1. *In vitro* phase ll glucuronidation in perch liver homogenate

The phase II reactions in which a conjugated metabolite of a xenobiotic is formed not only involve the xenobiotic or its phase I products, but also conjugating agents which are usually derived from carbohydrate, protein, or fat metabolism (**Table 5.1**). The six most common reactions are thought to be glucuronide formation, glycine conjugation, mercapturic acid synthesis, methylation, acetylation and ethereal sulphate synthesis. Five of these reactions have been demonstrated to occur in various species of fish by ***in vivo*** or by ***in vitro*** studies, the exception being mercapturic acid synthesis (Adamson, 1967; Adamson and Davies, 1973; Akitake and Kobayashi, 1975; Kobayashi *et al*, 1976; Kobayashi *et al* 1977; Layiwola and Linnecar, 1981).

These reactions are characterised by the occurrence of activated nucleotides as intermediates in the synthetic process and all require energy, usually provided by adenosine triphosphate (ATP). The nucleotide intermediate can involve either the activated conjugating agent or the activated xenobiotic.

The conjugated products formed in this way are generally less toxic than the original compounds and are nearly all relatively strong acids at physiological pH values. These reactions, therefore, tend to produce highly ionized, less lipid - soluble, non - toxic metabolites which are readily excreted by the organism and they are commonly referred to as detoxification mechanisms.

Table 5.1: Compounds involved in conjugation reactions in animals (Adamson and Sieber, 1975).

Source of conjugating agent	conjugating agent
Carbohydrate	Glucuronic acid (sometimes replaced by glucose, ribose or N - acetyl glucosamine)
Amino acid	Glycine (sometimes replaced by ornithine, arginine, glycyltaurine, glycylglycine, or serine)
	Glutathione (mercapturic acid synthesis)
	Methionine (methylation)
Miscellaneous	Acetyl (acetylation) sulphate
	(ethereal sulphate synthesis)

This chapter concerns studies whose central aim is to assess the impact that a hepatic parasite might have on the biotransformation of phenol by fish. Perch (*Perca fluviatilis*) liver infected with *Triaenophorus nodulosus* plerocercoids have been used for an ***in vitro*** biotransformation experiment. This was in anticipation that a comparison could be made between the ability of perch to biotransform phenol in the presence of parasites infecting the liver and that of sticklebacks with *S. solidus* plerocercoids in the peritoneal cavity.

5.2. Materials and Methods

5.2.1. Preparation of the sub - cellular fractions

Livers were quickly excised from both infected and uninfected perch (*Perca fluviatilis*) which had been killed immediately before by a blow to the head followed by decapitation. Each group of livers was pooled and washed with 0.25M ice - cold sucrose, blotted dry with filter paper and weighed. Each pool of four organs weighing approximately between 0.3 - 0.5g was then placed in a medium consisting of 7.8ml KCl (0.15M), 0.2ml $MgSO_4$ (0.16M) and 2ml sodium phosphate buffer (0.1M, pH 6.8). to make up a 10% homogenate (Layiwola, 1988). All solutions were kept at 4°C. The homogenisation was carried out in a vessel constructed of a borosilicate glass tube with a stainless steel pestle capped with PTFE (Jencons, Leighton, Beds, U.K.) rotating at 2000 rpm with up and down strokes for one minute. The motor of the homogeniser was a Citenco type DTS 7333 (Park Products, Blackburn, England). The prepared liver homogenates were transferred to 50ml polycarbonate centrifuge bottles and placed in a bench - top, high - speed centrifuge which was programmed to operate at the desired speed to produce an acceleration of 10,000g (MSE, Centaur 2). The centrifugation step was set to continue for a period of 10 minutes in order to obtain a mitochondria - free supernatant liquid. The supernatants were retained and the resultant, residual pellets were discarded. All incubation experiments used the mitochondria - free supernatant.

5.2.2. Incubation procedure with phenol

Samples of 2ml of the mitochondria - free supernatants were aliquoted into 10ml conical incubation vessels (Pyrex®, England). To each aliquoted incubation medium, phenol (0.2ml, mg/ml) and the donor compound, uridine di - phosphate glucuronic acid (UDPGA) (0.5ml, 10mg/ml) were added. Samples were incubated at 37 °C for 1h in 10 ml glass stoppered conical flasks and shaken in a Dubnoff metabolic water bath. The reaction involving the phenol donor molecule and the liver homogenate was terminated by the addition of 10% sodium tungstate (0.5ml/incubate), a protein precipitant. Incubate volumes were then adjusted to 5ml using hplc - grade water (Fisons, Loughborough,

Leicestershire, England) and the precipitated proteins were "spun - out" in the bench - top centrifuge operating at 4000g for 10minutes.

5.2.3. Estimation of phenyl glucuronide

Samples of the protein free incubates were analysed using hplc as indicated in **Chapter 2.** The internal standard method described and employed in the stickleback experiments was used to determine the amount of the phenyl glucuronide and parent compound present.

5.3. Results

5.3.1. *In vitro* biotransformation of phenol in uninfected and*T.nodulosus* - infected perch liver incubates

Using the liver homogenates from non - parasitised and parasitised fish a series of ***in vitro*** incubation procedures was performed, involving phenol as the toxicant **Figure 5.1** represents typical chromatograms of the ***in vitro*** phenol biotransformation. The glucuronide conjugate was identified by its retention time of 1.47 - 1.49*. Identity was confirmed by the use of β - glucuronidase hydrolysis on the samples after which the conjugate peak decreased in size and the parent peak with a Rt of 3.40 - 3.49* increased in peak size as shown in **Figure 5.2**. Both the uninfected and infected fish exhibited the ability to synthesise glucuronide from phenol. This suggests that liver infection by *T. nodulosus* does not completely abolish the glucuronidation ability of the fish liver in the incubation medium.

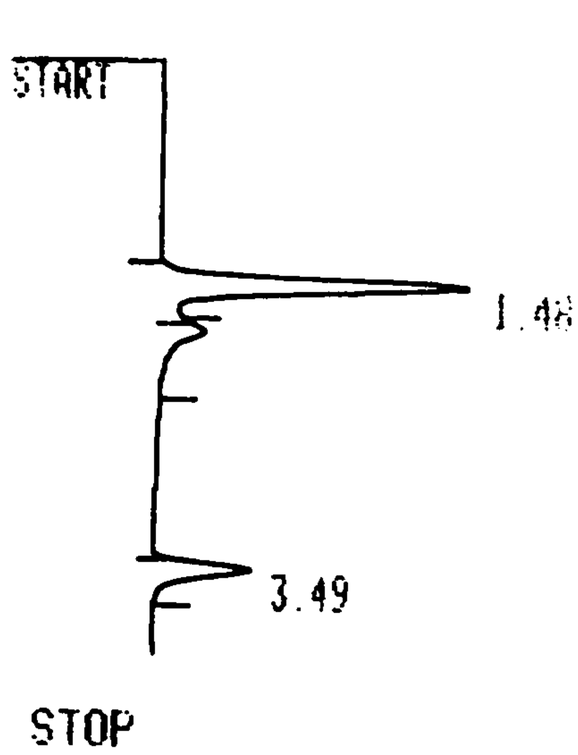
The amounts of phenyl glucuronide and phenol recovered through the internal standard method are shown in **Table 5.2**. There appeared to be no significant difference in the amounts of parent compound and metabolite recovered in parasitised and non - parasitised fish. 8.6µg/ml of phenylglucuronide was recovered from the parasitised liver while 8.2µg/ml came from the non-parasitised liver.

* min

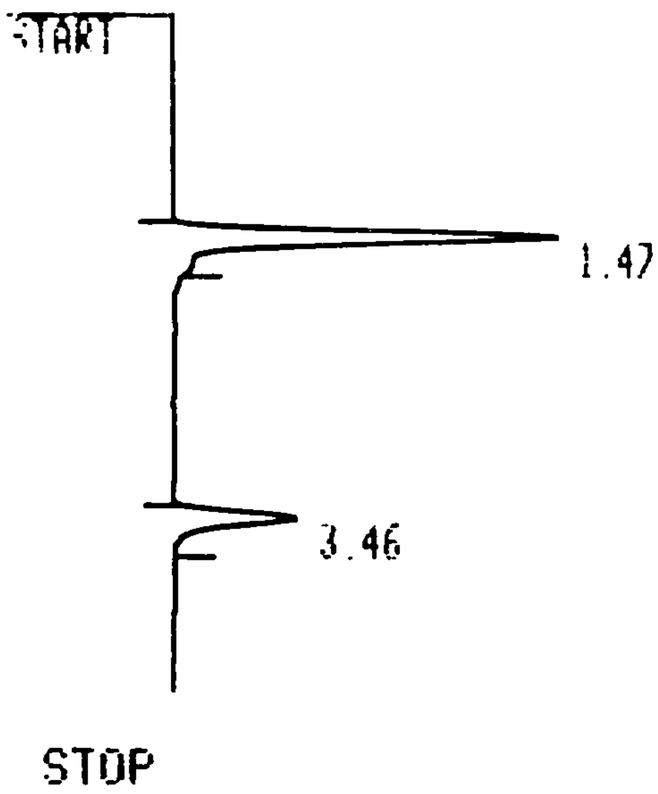
Table 5.2: Amounts of phenyl glucuronide and phenol obtained from Liver homogenates.

Unit : μg/ml (mean ± S.E, N=3)

Compounds	Non - parasitised	Parasitised
Phenyl glucuronide	8.2 ± 0.2	8.6 ± 0.3
Phenol	1.2 ± 0.2	1.9 ± 0.2

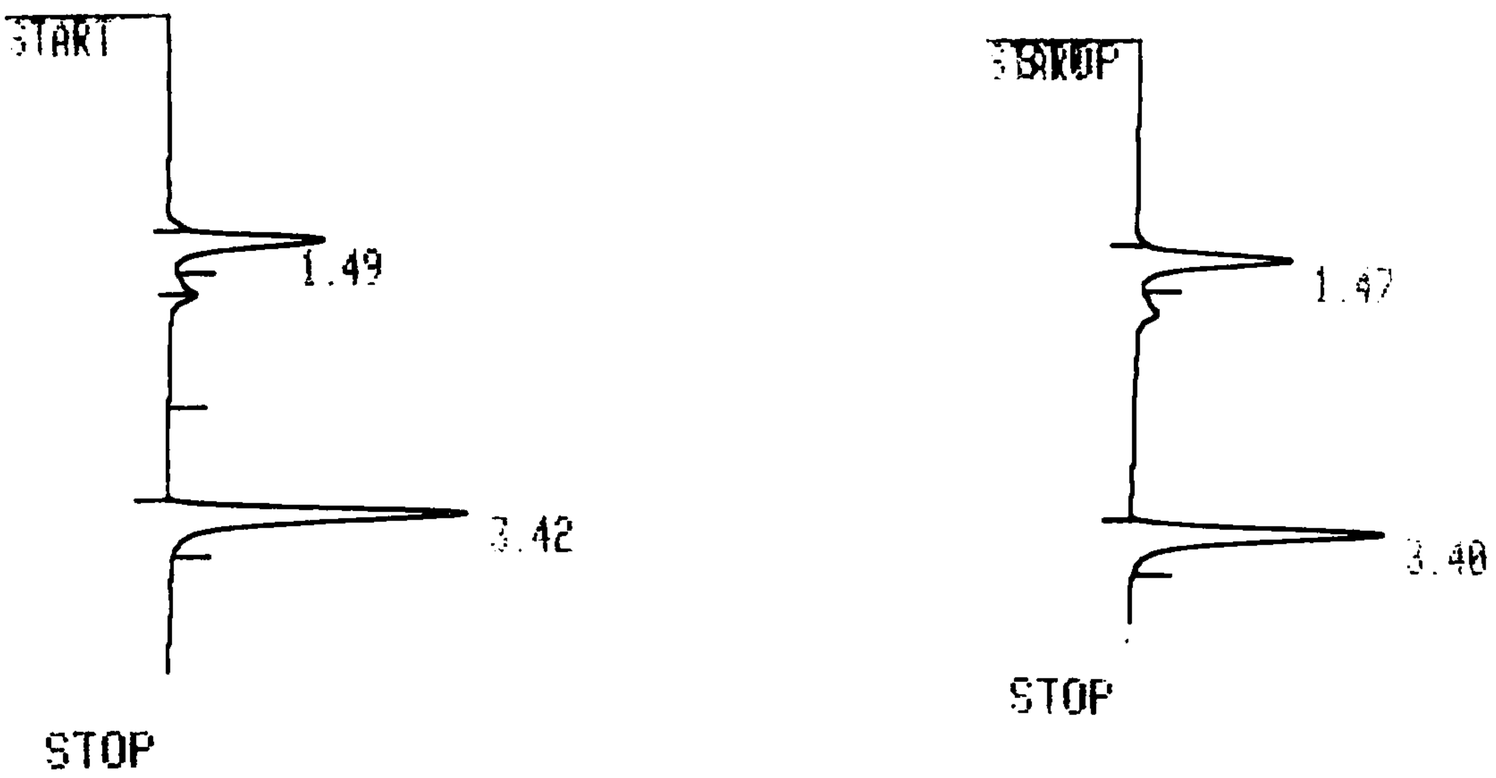


Non - parasitised



Parasitised

Figure 5.1: Hplc chromatograms of the *in vitro* phenol biotransformation in the liver of non - parasitised and parasitised perch (*Perca fluviatilis*).



Non - parasitised **Parasitised**

Figure 5.2: Hplc chromatograms showing the enzyme hydrolysis of phenyl glucuronide and the relative increase in the peak associated with the parent coumpound in the liver homogenate of the non - parasitised and parasitised perch.

Evidence from **Section II** of this chapter, with respect to the gross pathological changes in the liver, indicates that there was a considerable degree of liver fibrosis and large areas of necrosis associated with *Triaenophorus* plerocercoids in the liver. It is interesting to note given the level of parasite infection and tissue damage, that there was apparently no significant reduction in the biotransformation activity.

Section II

5.4. Histopathological investigation of liver plerocercoids of *Triaenophorus nodulosus* in perch, (*Perca fluviatilis*)

The genus, *Triaenophorus*, is broadly represented by three key species, *T. nodulosus*, *T. crassus* and *T. stizostedionis*, all of which are resident in the liver and enteric tissues of fish species. What is more, these parasites exert a significant pressure on many larger important species including, pike (*Esox lucius*), Atlantic charr (*Salvelinus alpinus*), brook charr (*Salvelinus fontinalis*), perch (*Perca fluviatilis*), tench (*Tinca tinca*) and bream (*Abramis abrama*) (McKinnon and Featherstone, 1982; Hoffmann *et al*, 1986 and Shostak and Dick, 1986).

In the life cycles of pseudophyllidian cestodes in the genus *Triaenophorus* the final host is commonly the pike, *Esox lucius*. Pike become infected by consuming second intermediate host fish in which plerocercoids are contained. In the case of *T. nodulosus* this second intermediate host is usually the perch, *Perca fluviatilis,* and the plerocercoids are typically found in the liver.

Numerous studies have described intestinal, hepatic and renal pathology induced by both plerocercoids and adults of *Triaenophorus* in pike, *Esox lucius*, and perch, *Perca fluviatilis*. Such observations on gross pathology have ranged from no or limited damage (Miller, 1943; Bucke, 1971; Mackiewicz *et al*,1972; Kuperman, 1973; Pronina and Pronin, 1982) to situations where chronic inflammation and fibrosis have been evident in gut and liver tissues, particularly at the point where the scolex of the parasite had become

deeply embedded in the tissue (Williams, 1960; Mackiewicz *et al*, 1972; Kuperman, 1973; Korting, 1977; McKinnon and Featherston, 1982).

Shostak and Dick (1986) have reported gastrointestinal histopathology of pike infected by *T. crassus*. Extensive inflammation was reported in the intestine of the pike with severe ulceration of the mucosa being observed. The appearance of numerous lesions and of opacification were noted which were due to tissue necrosis and the deposition of a considerable amount of collagen during the subsequent tissue regeneration. Additionally, these lesions were well vascularised and haemorrhaging within these areas was a frequent occurrence.

The cestode *T. nodulosus*, in common with its counterpart *S. solidus*, has a first intermediate host which is typically a copepod. As many as 72 fish species have been reported as being the second intermediate host for *T. nodulosus* and the plerocercoids of this parasite have been found in large numbers in the livers of these species (Hoffmann *et al*, 1986).

Hoffmann *et al* (1986) have reported that the plerocercoids of *T. nodulosus* cause extensive damage to the fish liver. In a study involving perch (*Perca fluviatilis*), burbot (*Lota lota*), Atlantic charr (*Salvelinus alpinus*) and minnow (*Phoxinus phoxinus*) they observed many examples of encapsulated plerocercoids in the liver. They further noted that in charr and minnow only live plerocercoids could be found, whereas in burbot and perch, dead and destroyed plerocercoids were also present which were accompanied by areas of fibrosing tissue on the surface of the liver.

Liver pathogenesis as marked by the formation of collagenous plaques, was also considered to be of significance in relation to *T. nodulosus* infection in both the perch and the burbot. This theme has been further developed within the scope of this current investigation with specific reference to perch (*Perca fluviatilis*). The presence of epithelial cell granulomas around parasites has given rise to the hypothesis in this study that whole liver dysfunction may ensue following infection with *T. nodulosus* and that this may be a precursor to relative hepatic failure in fishes.

In infections with *Triaenophorus*, many fishes experience manifest alteration of liver morphology. Extensive changes in the deposition and distribution of collagen have been noted. In many necropsied fish, it is evident that plerocercoid infection causes hepatic haemorrhage and changes in liver vascularisation. In most fishes, such as the pike examined by Shostak and Dick (1986) the gut was shown to be highly fibrosed and in some instances there was evidence of severe tissue necrosis, especially in more advanced levels of infection. Staining techniques have further demonstrated that there is extensive deposition of collagen and fibrin at the areas of liver closest to the hooks of the parasites (Hoffmann *et al* 1986).

The aim of this section of the present chapter is to assess the extent of modification of liver tissue which resulted from the infection caused by the cestode, *T. nodulosus*, and to evaluate the possibilty of developing a simple volumetric model that may link the effect of the hepatic parasite with the toxicity and biochemical metabolism of environmental contaminants in fish.

5.5. Materials and Methods

5.5.1. Staining techniques of the normal and parasitised liver perch

5.5.1.1. Haematoxylin and Eosin. (H and E)

Sections of normal and parasitised liver were stained with H and E by adding two aliquots of xylol to the sections over two consecutive 5 minute periods. The sections were then treated with 100% then 70% ethanol. Haematoxylin staining solution was then added to the sections for 10 minutes. The sections were then washed with alkaline tap water for 5 minutes. Acid - alcohol (1%HCL in 20% ethanol solution) was then added to the sections for 5 -10 seconds. The sections were then promptly rinsed with distilled

water. A solution of 1% eosin was added in 70% ethanol and left for 3 minutes. The sections were then subjected to treatment with 90% ethanol for a further 15 minutes

followed by 100% ethanol for two further 2 minutes periods. Xylol was then, finally, applied for 2 minute and the sections were then mounted by Ralmount in xylol.

5.5.1.2. Mallory - Trichrome stain

Mallory - Trichrome stain was used for staining musculature and collagen. The section of livers were treated with xylol for two 5 minute periods. The sections were then prepared in 100% followed by 70% ethanol for 2 and 1 minutes respectively. Washing was performed using distilled water. One % acid fuchsin stain was then added for 5 minutes, followed by rinsing with distilled water for 3 - 4 minutes. Mallory - Trichrome stain (comprised of 1% alcian blue and 50% Orange C in distilled water) was then applied for 20 minutes. Following application of the stain, the section was further treated with two application of 95% ethanol and then a further two application of 100% ethanol for 2 x 5 and 2 x 2 minute periods respectively. Xylol was then added for 2 minutes followed by a further 1 minute application. Stained muscle tissue appeared red and collagen was stained dark blue following the use of the stain.

5.5.1.3. Alcian Blue stain for mucopolysaccarides

Sections were treated with xylol for 5 minutes and this phase was repeated. The sections were then placed in 100% ethanol for 2 minutes followed by treatment with 70% ethanol for a further 1 minute. Sections were then twice washed with distilled water. Alcian blue stain was then added to the sections. Washing was then repeated three times with distilled water. A solution of 1% periodic acid was then added to each section and this was applied for a duration of 5 minutes. Schiff's stain was then applied to the sections for 15 minutes. Sections were then washed with running water for 10 minutes. Distilled water was then used to wash the slides for a period of 1 minute. Additions of ethanol, comprising of 70% , 70% then 100% respectively, were then added to each sections. The sections were further treated with xylol for 2 minutes (in 1 minute aliquots) then mounted for examination. The presence of acid mucopolysaccharides were noted by the formation of a blue colouration whereas neutral mucopolysaccharides were typified by a red colour.

5.5.2. Stereological analysis of histological section

Areas of fibrotic and undamaged liver tissue (unstained and pink respectively in PAS - Alcian Blue Stained Section) were estimated by cutting appropriate areas from micrographs and weighing them. Ratios of areas were assumed to be equivalent to ratios of volumes, hence masses.

5.5.2.1 Results

Figures 5.3 - 5.12 illustrate the normal histological appearance of non - infected perch livers and the range of histopathological changes associated with the presence of plerocercoids of *T. nodulosus* in the liver.

Several generalisations emerge from the histopathological information provided by these specimens, namely:-

(i) Uninfected livers are largely composed of columnar arrays of hepatocytes which stain PAS positive, almost certainly because of the presence of the storage polysaccharide, glycogen, in such cells.

(ii) Plerocercoids are characterised by Alcian - blue positive surfaces presumably linked with mucopolysaccharides associated with the outer layers of the cestode tegument and the tegumentary glycocalyx.

(iii) The zone of liver tissue immediately surrounding each plerocercoid is associated with inflammation (white blood cell infiltration), fibrosis (probably collagenous) and a lack of PAS positive material.

5.2.3. (b) Stereological evaluation of the extent of liver fibrosis and changes in gross tissue pathology in perch (*Perca fluviatilis)* in moderately and highly infected liver by *T. nodulosus*

The **Figures 5.3 - 5.12** show a quantitative assessment of the degree of tissue fibrosis and necrosis experienced by small and large livers. By assessing the nature of the sites occupied by the *T. nodulosus* and the nature of the infection in the perch, it has been possible to describe the ratio of "normal" liver to that experiencing fibrosis. Careful histological measurements, primarily of differential areas of fibrotic and normal liver tissue, were made from the micrographs (see **section 5.2.2**). These, in turn, were equated with the weights of fibrosed and undamaged tissue. This exercise proved useful in ascertaining the full extent of the zones of liver that were subjected to pathological changes.

Table 5.3: A summary of the tissue pathology, and stereological measurements obtained from the uninfected, moderately and highly parasitised livers (uninfected liver = 0 cestode, moderately infected liver = <10 cestode, and highly infected liver = >10 cestode)

	Uninfected liver	Moderately infected liver	Highly infected liver
Weight of the liver (g)	0.07	0.26	0.6
Unfibrosed area estimates	100% 100% 100% 100%	24.17% 26.08% 26.21% 24.58%	19.77% 17.64% 17.24% 18.56%
Unfibrosed mean area estimate	100%	25.26%	18.30%
Unfibrosed volume estimate	100%	25.26%	18.30%
Estimate of weights of unfibrosed liver tissue	0.070	0.066	0.1098

Table 5.3 summarises the stereological analyses carried out in this study. The results for each of three livers (one unparasitised, one moderately parasitised and one heavily parasitised) are presented in terms firstly of relative areas of fibrotic and normal liver

tissue. These are then converted into mass estimates using the known weights of the livers. The Table makes it clear that total liver mass increases dramatically on infection.

Further data from, in total, 6 uninfected and 11 infected liver confirms this impression. For those livers the mean weight of uninfected livers was 0.058g (range 0.05 - 0.07g) while that of infected livers was 0.229g (range 0.08 - 0.60g), all from fish in the weight range from 3.9g to 4.8g.

Despite this increase in weight the stereological analysis makes it obvious that the great majority of this infection - related increase is caused by new fibrotic tissue. The weight of unfibrotic, presumably normal liver tissue changes very little between the three livers, with values of 0.070g (uninfected), 0.066g (moderately infected) and 0.110g (heavily infected).

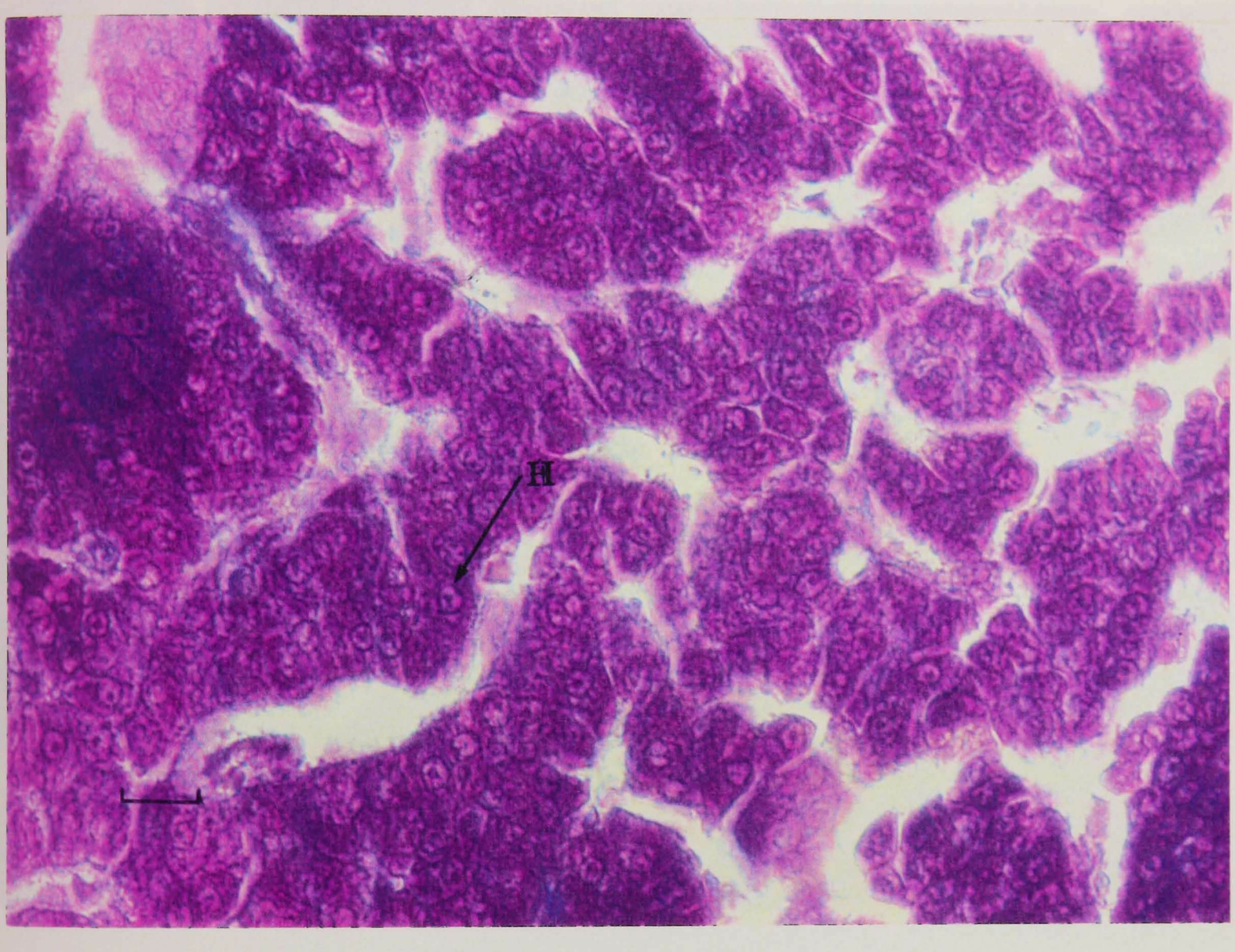


Figure 5.3: A section through a normal, uninfected perch liver (Mallory stain). This section demonstrates intense basophilic staining of hepatocyte cytoplasm and shows a series of columns of polygonal hepatocytes each with a large round nucleus containing a single nucleolus. (H = hepatocytes; scale bar = 100μm).

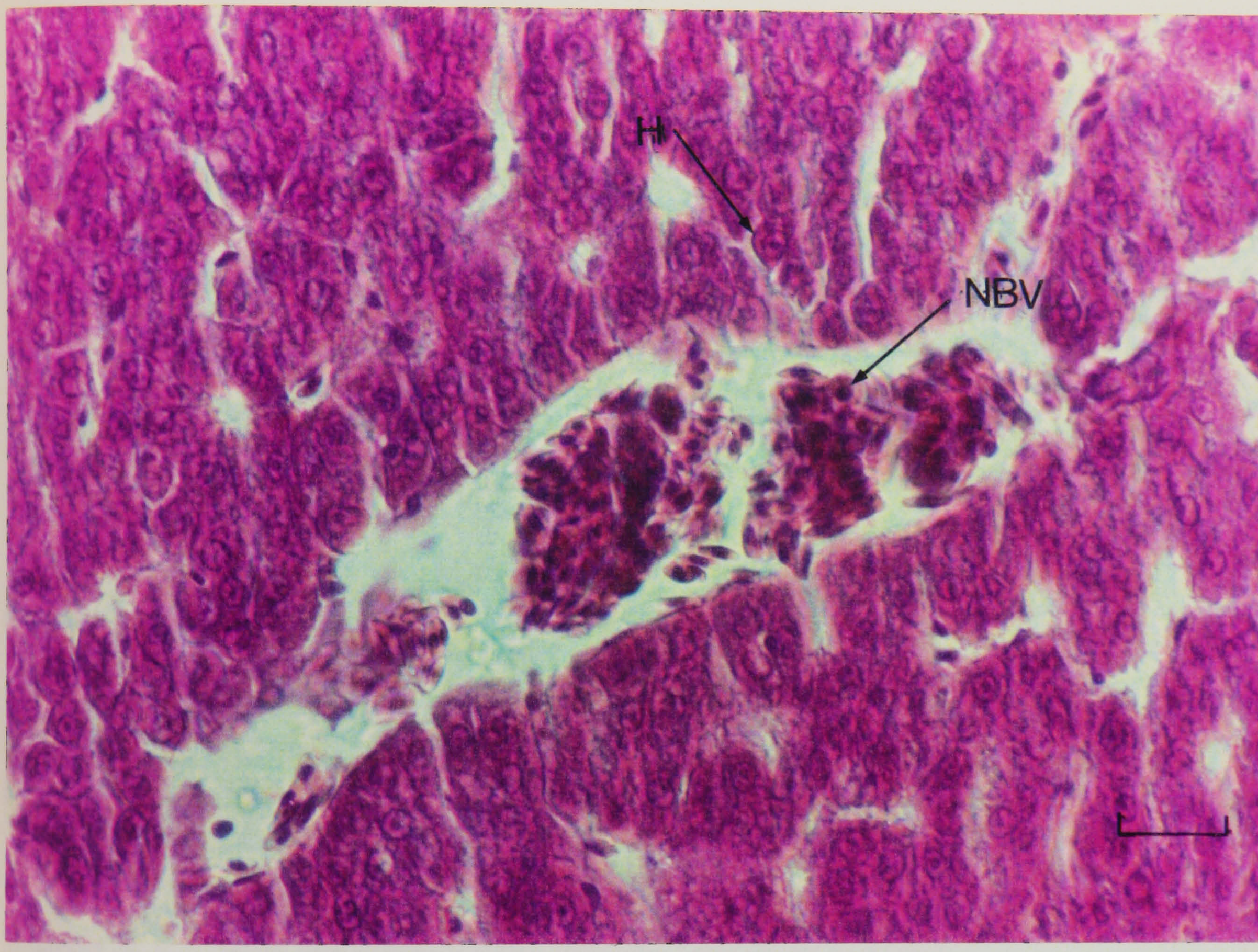


Figure 5.4: Uninfected perch liver (Haemotoxylin and Eosin stain). Columns of hepatocytes are very evident and there is a longitudinal section through a hepatic blood vessel containing nucleated red - blood cells. (H = hepatocytes; NBV = normal blood vessel; scale bar = 100μm).

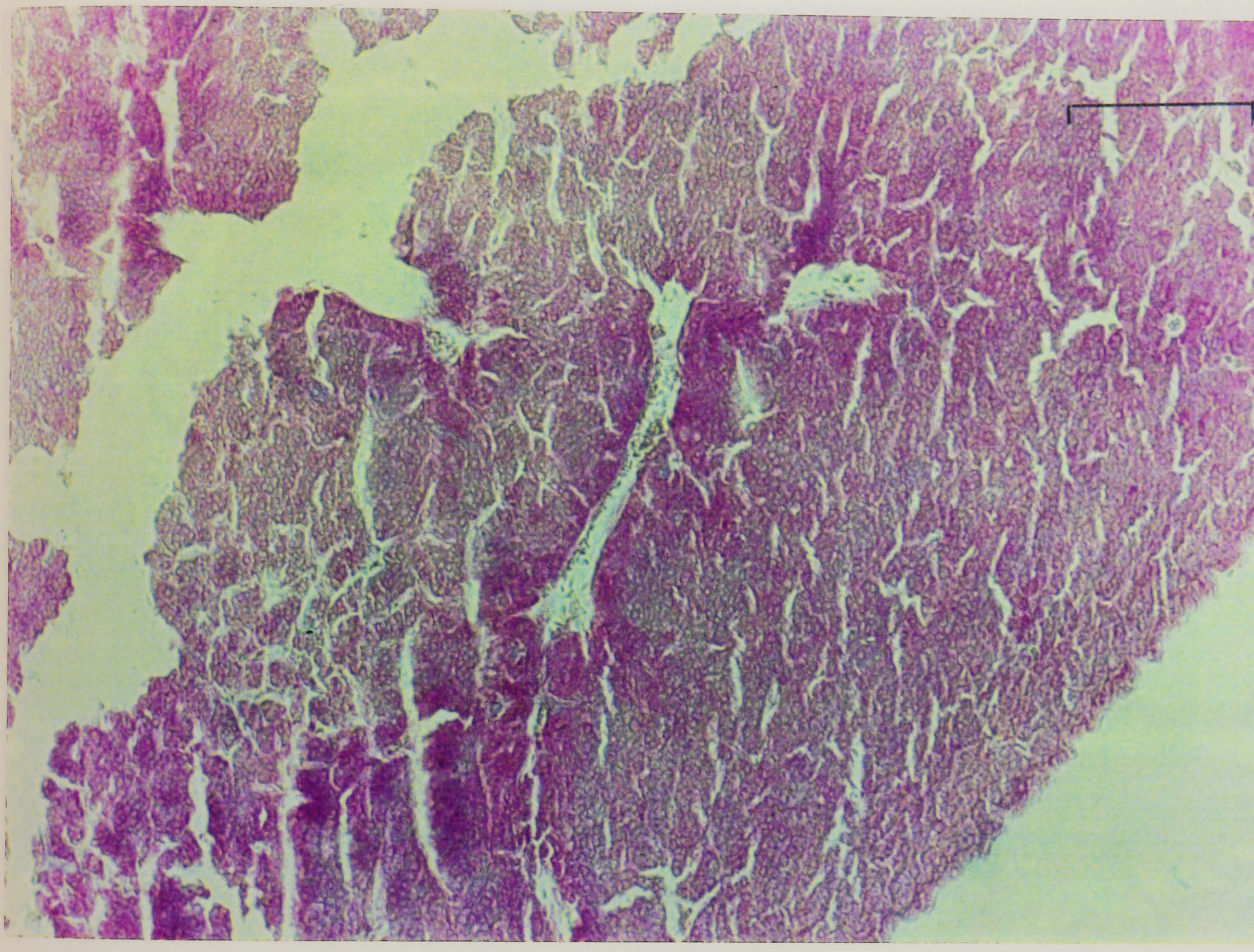


Figure 5.5: Uninfected perch liver (PAS/Alcian Blue stain). This section reveals generalised PAS positive staining (pink) in most hepatocytes. There is no significant Alcian Blue - positive staining. (scale bar = 100μm).

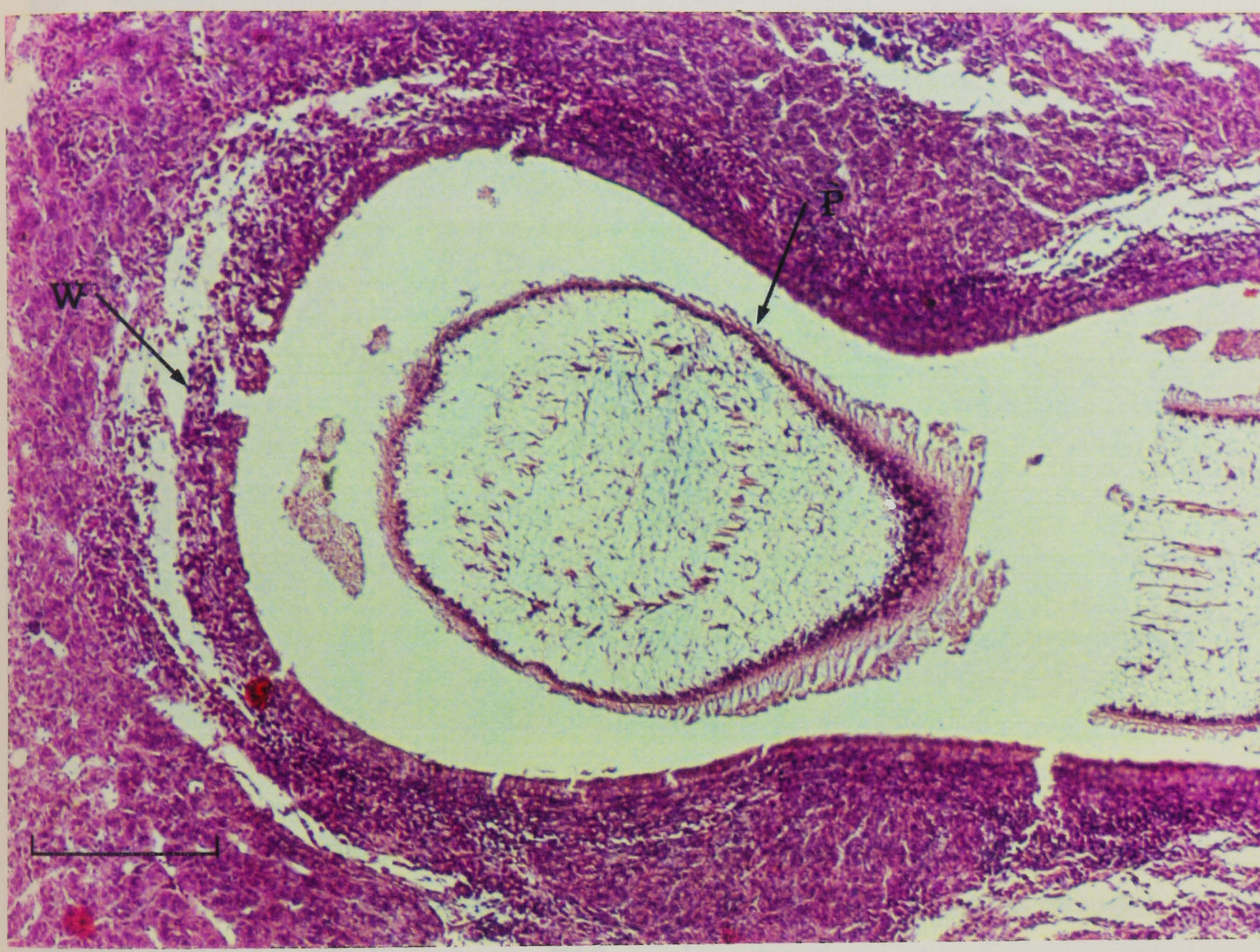


Figure 5.6: Perch liver with moderate level of plerocercoid infection (H and E staining). P = plerocercoid; W = white blood cell infiltration (inflammation), (scale bar = 1mm).

Figure 5.7: Perch liver with moderate level of plerocercoid infection (PAS/Alcian Blue stain). This low - power image through an infected liver shows sections through a number of plerocercoids, layered non - PAS - positive tissues around each parasite and zones of more normal pink staining PAS - positive hepatocytes in regions further away from the parasites. Each of the sections through the plerocercoids shows an intense blue staining (Alcian blue positive) at the periphery. (H = hepatocytes; P = plerocercoid; scale bar = 1mm).

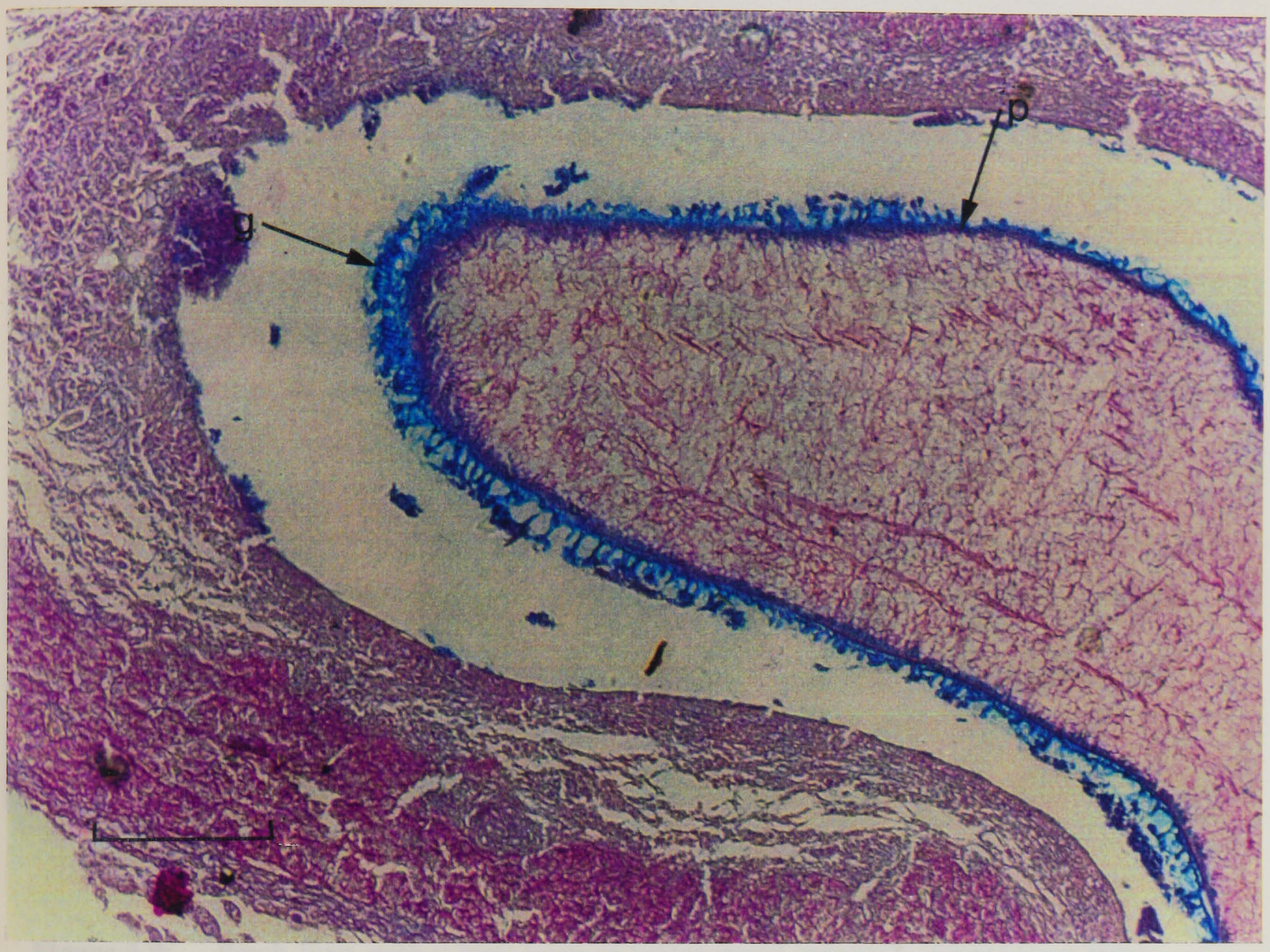


Figure 5.8: Perch liver with moderate level of plerocercoid infection (PAS/Alcian Blue stain). This image illustrates clearly the difference between PAS -ve and PAS +ve regions, adjacent to, and distant from, the plerocercoid respectively. It also shows the AB +ve staining residues in the tegument and presumably the glycocalyx of the parasite. It probably results from the presence of mucopolysaccharides in these locations. (p = plerocercoid; g = glycocalyx; scale bar = 100μm).

Figure 5.9: Perch liver with a high level of plerocercoid infection (PAS/Alcian Blue stain). A parasite profile is shown in this image, which has been stained with PAS/Alcian blue. Surprisingly, there has been markedly less tissue shrinkage in this preparation than has been experienced in other examples of histology. This indicates that for about approximately half of the parasite's circumference it is indirect contact with the surrounding liver tissues, with this contact zone revealing a high intensity of Alcian blue +ve staining. The fibrous zones around each parasite are PAS -ve. (P = plerocercoid; H = hepatocyes; scale bar = 1mm).

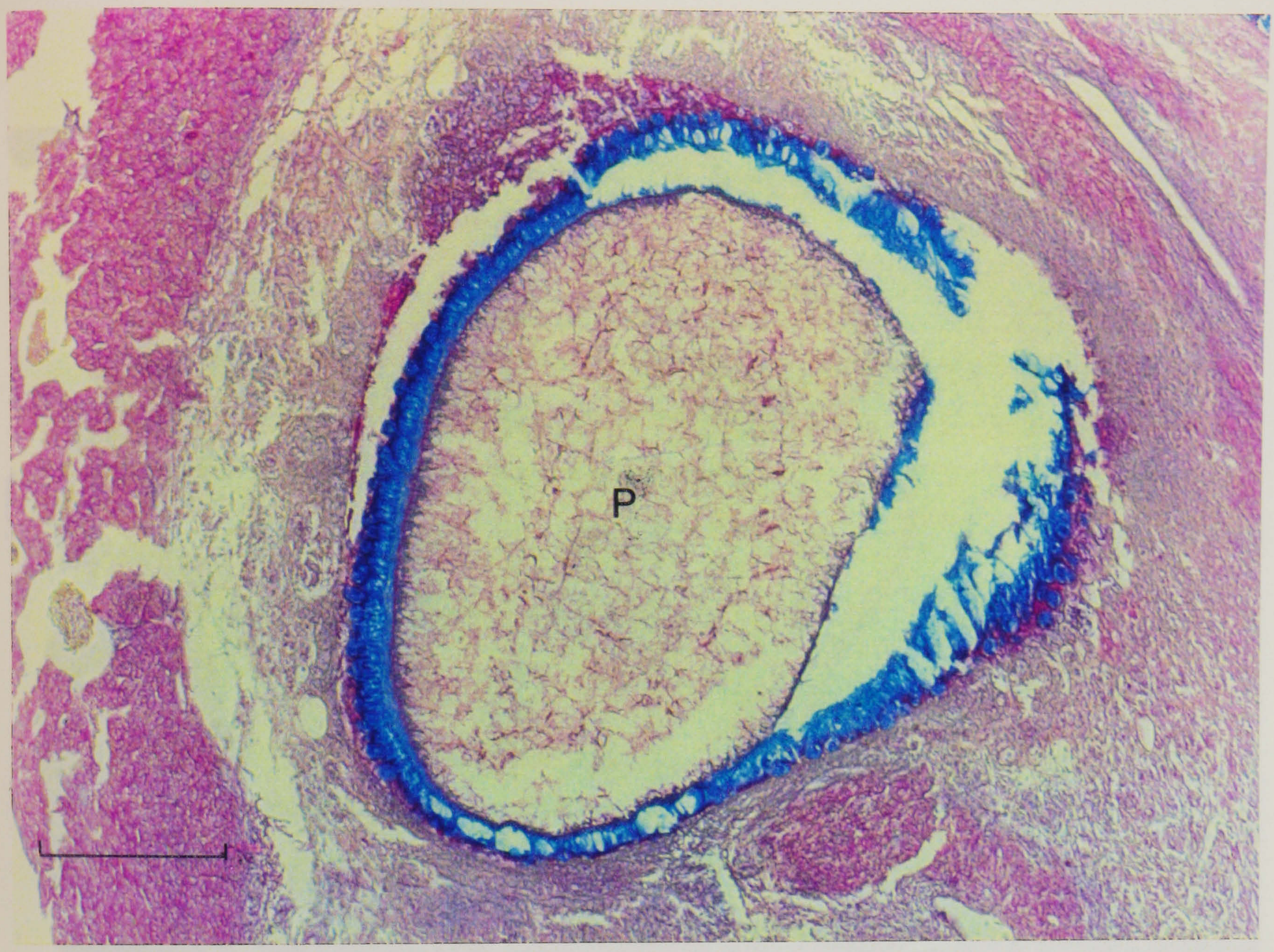


Figure 5.10: In this image, a significant portion of the contact zone between the parasite and the surrounding liver is highlighted and consolidates the histological observations made in **Figure 5.9.** There is some PAS +ve staining in the subintegumentary zone of the plerocercoid (almost certainly attributable to staining within the body wall and musculature of the parasite) and in the liver tissue immediatly adjacent to the parasite. The most intense staining in the image is the Alcian blue (AB) +ve material in the parasite tegument and in the glycocalyx zone immediately distal to the tegument. (p = plerocercoid; scale bar = 1mm).

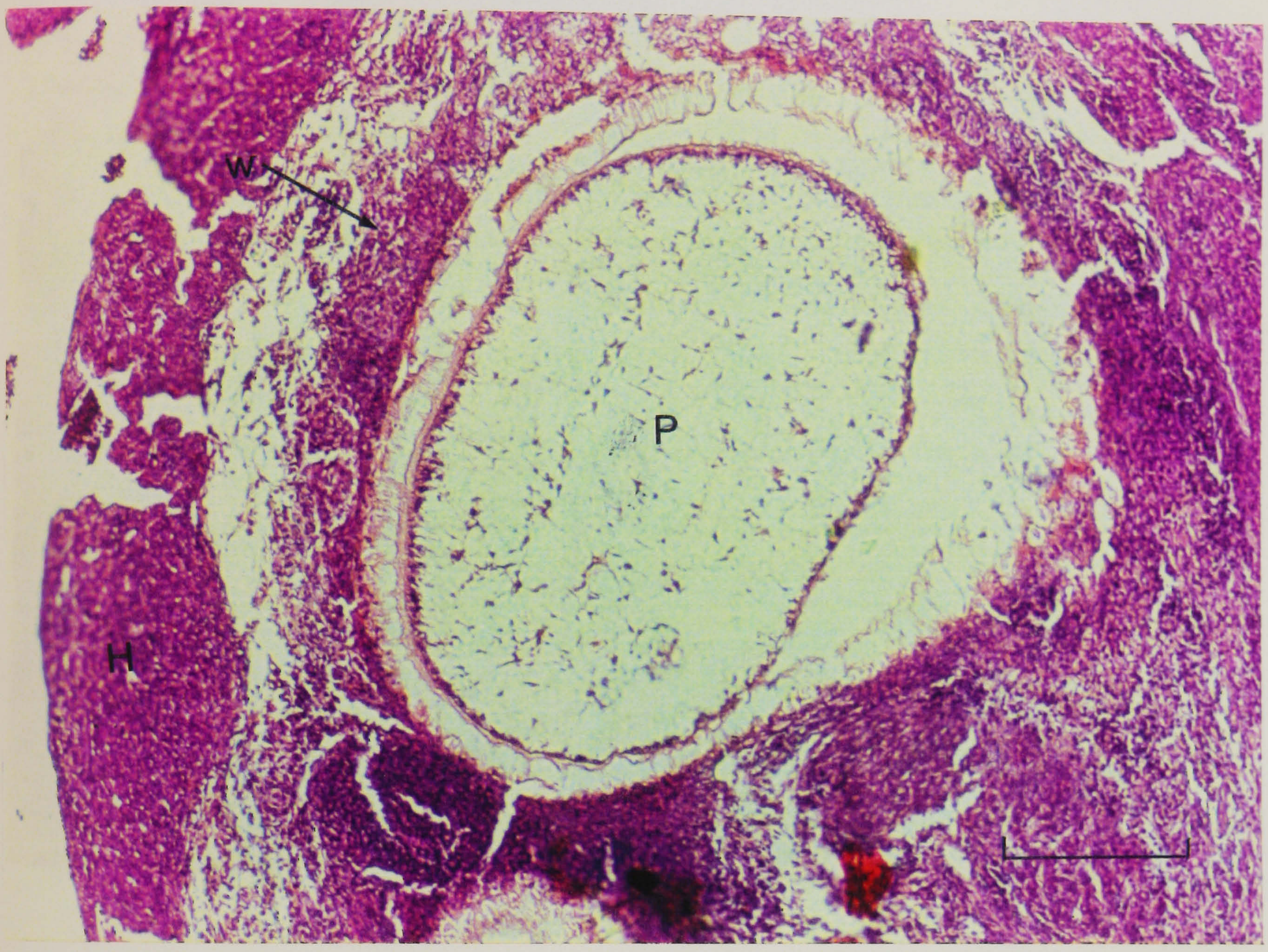


Figure 5.11: This section, stained with H & E stain, corresponds to that shown in **Figure 5.10**. Distinct staining of the nuclei with H & E shows both an intense white cell infiltration in the liver tissues around the parasite and the parasite nuclei of the tegumentary cell bodies and the body wall muscles in a sub - tegumentary location. (w = white cell infiltration; p = plerocercoid; H = hepatocytes; scale bar = 1mm).

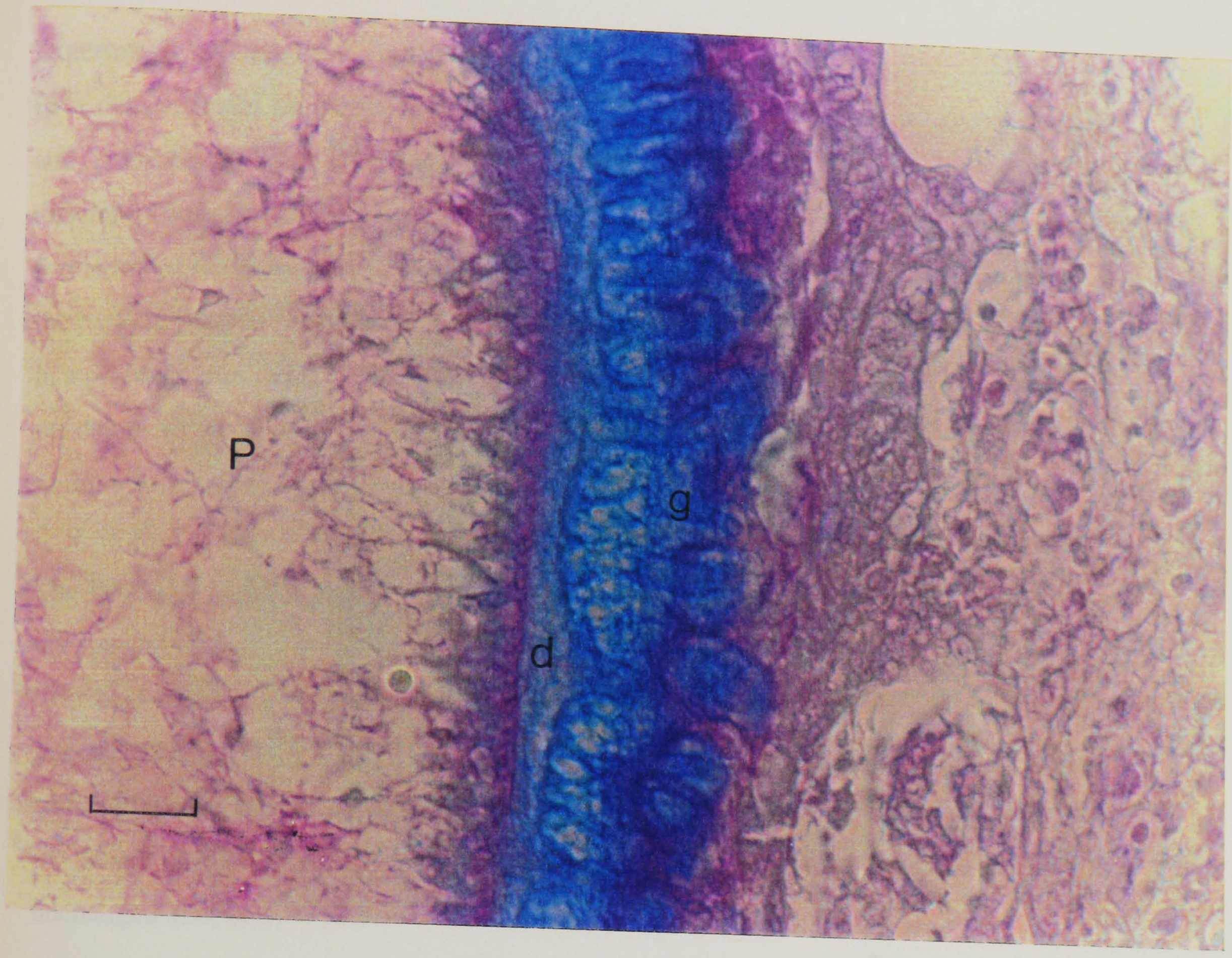


Figure 5.12: This section, stained with PAS/Alcian Blue shows a detail from **Figure 5.9**. It reveals the interface zone between the surface of a plerocercoid and the surrounding liver tissue. The most intense staining in the image is the Alcian Blue +ve material at the surface of the parasite corresponding to the distal cytoplasm of the parasite tegument and its glycocalyx. (P = plerocercoid; d = distal cytoplasm of tegument; g = glycocalyx zone; scale bar = 100μm).

5.6. Discussion

Experimental data derived from a series of ***in vitro*** experiments, in **Table 5.2** (section I) have demonstrated the differences between the rates of glucuronide formation in infected and uninfected liver. It is apparent, from this data, that there was no appreciable difference between the parasitised and non - parasitised liver samples in terms of the formation of the phase II glucuronide entity. In fact, the level of glucuronide formation in the parasitised liver was slightly elevated relative to the non - parasitised sample although in a probably non - significant way. This regarded as being somewhat unexpected, in that the degree of fibrosis in the parasitised liver was high and there was a significant element of non - functional tissue.

The lack of a significant change in phase II activity in the parasitised perch liver is an unexpected finding. **Table 5.3** (section II) indicates that the weight of unfibrotic normal liver tissue changes very little between the uninfected, moderately infected and heavily infected fish, with the great bulk of liver weight increase presumably being due to the parasites themselves and to new fibrotic tissue. If the remaining normal liver tissue has an unchanged weight it is, on first analysis, not surprising that phase II activity is unchanged after parasitisation.

In fact, as the standard sample of liver used to assess biotransformation in infected fish included both parasites and fibrotic material, the fraction of the standard sample which was normal would be expected to decline. This would be expected to produce a reduced biotransformation level. The fact that this did not happen, suggests one or more of the following explanations :-

(i) the parasites themselves can carry out biotransformation

(ii) collagenous tissue can carry out biotransformation

(iii) the normal liver tissue in an infected liver has enhanced biotransformation capacity.

In this brief experimental investigation it was hoped that the link between histopathological change and biotransformation could be identified. The experiments

here have not been as conclusive as anticipated and this is where there exist openings for future research that will :-

(i) develop an appropriate regime that will adequately link disease and biotransformation in fish liver

(ii) consider the contribution of parasitic biotransformation in the diseased state and

(iii) evaluate the suitability of hepatic biochemical parameters as indicators of the effects of pollutants on diseased livers.

This preliminary investigation, whilst negative in its major findings, has at least set the stage for future research.

The relationship between the ***in vitro*** Phase II biotransformation and interference of parasite could not be established as normal and *T. nodulosus* - diseased fish both have an ability to biosynthesise glucuronide. There is the probability that the selection of the ***in vitro*** methodology may have been inappropriate and an ***in vivo*** exposure route may have yielded more convincing data. However, this proposition needs to be tested with caution as the ***in vivo*** experiment of the stickleback did not establish any difference.

Perhaps, after all, the site of infestation may not have any major part to play in the toxicity and biotransformation of phenol to fish.

Chapter Six

General Discussion.

Risk analysis of organic chemicals in aquatic systems requires information on their dose - response relationships to the endogenous populations and the levels of exposure that are likely to be encountered. From this it can be ascertained if exposure for a particular chemical is above or below the onset of adverse effects.

In such studies toxicity data are obtained from laboratory experiments with specially reared organisms and parasite infection is usually minimal but in the ambient environment such as the River Wandle the opposite is the case. As this and previous work has shown, the populations of sticklebacks in the Wandle are infected with variety of ecto - and endoparasites. This raises the question as to whether these populations are more or less vulnerable to toxic chemical exposure. This is one of the basic questions underlying the present study and it was decided to base the investigations on the cestode parasite *S. solidus* as a model system.

From this emerged the four primary objectives of the study. The first of these required the investigation of stickleback populations in the River Wandle in relation to the patterns of parasite infection. The second was to correlate these patterns with available data on pollution levels in the different sections of the river. The third and fourth objectives were to evaluate the toxicity of phenols in different host - parasite models.

The study of sticklebacks in the River Wandle demonstrated a complex pattern of infection with various species of parasites in that there were parallels and differences with those of other studies. It was concluded that the complexity of these relationships made it very difficult to draw any simplistic conclusions about links between types and levels of parasitic infection and levels of environmental contamination. It will be recalled that marked differences in infection with *S. solidus* at sites B and E were found. This was despite the fact that these sites are less than 2

km apart. It was not possible to ascertain the reason for the difference in infection levels between the two sites, but it is clear that some factor or factors have a retarding influence on the growth and development of *S. solidus* at one site or an enhancing one at the other. It would appear that these differences are not due to differences in metal contamination between these sites. Although it is possible that the factorial difference between the two sites is the presence of an unidentified deleterious environmental factor at site E, recent studies by Klein *et al* (1995) mean that at least one additional possibility has to be taken into account. These studies have demonstrated that standard evaluation procedures of hazardous chemicals in the environment do not facilitate identification of mechanisms which might modulate potentially harmful effects. The modulation studies by Klein *et al* (1995) was that related to the presence of organic geochemical matrices such as humic substances. They discovered that the addition of humic geochemical matrices to standard *Daphnia* and fish tests increased or decreased the bioconcentration or toxicity of test substances such as substituted phenols and anilines in an unpredictable way. These results generate a different type of hypothesis in which reduction of parasite growth and development is being caused by a different sedimentary geochemical matrix at E which makes one of the previously discovered chemicals more toxic via increased bioavailability. It is certainly the case that the nature of the sediment in which xenobiotics are found influences their availability to fish and their capacity to biotransform them (Djomo *et al*, 1996; Qiao and Farrell, 1996).

The present study has demonstrated that a range of different parasites in sticklebacks show quite different responses (in term of infection levels) to the precise location of the hosts in the river system, with the localised differences in external environmental factors (including toxins) that these locations imply. These differences in the responses of different parasites suggest that it will be very difficult to use changes in parasite load in a direct and simple way as fish biomarkers for the presence of xenobiotics. Those biomarkers which have been proposed including enzyme alterations, bile metabolism, RNA/DNA ratio, skeletal abnormalities and histopathological lesions (see Holdway *et al*, 1995) usually show a more clear - cut

association with an external xenobiotic than the variable parasite infection responses demonstrated in the present study.

The high frequency of infection by *S. solidus* in sticklebacks and the fact that it can be easily observed in live fish provided a useful model in which to test the hypothesis that infected fish were more susceptible to phenol and pentachlorophenol. The literature referred to in Chapter 1 (Tierney *et al,* 1996; Milinski, 1984, 1990; Giles, 1987; Walkey and Meakins 1970) strongly indicates that *S. solidus* is a highly efficient converter of energy and can use substrates much more effectively than its host. It seemed likely therefore that the parasite in diverting resources in this way would make the host more susceptible to toxicants and this could be a consequence of a diminished capacity to metabolise and hence excrete the toxicants.

The present study has found no evidence for this. The experiments reported in **Chapter 4** strongly suggest that *S. solidus* infection of sticklebacks has no influence on the toxicity of phenols to the host fish. Similarly from the work on the phase II products of phenol metabolism it would appear that the parasite had no observable influence on this type of biotransformation. The work with the phase I and phase II inhibitors was inconclusive and in hindsight it is not clear whether the doses were sufficient to achieve the required inhibition.

It was felt that the perch/liver parasite(*Perca fluviatilis/T. nodulosus*) model provided a more direct test of the hypothesis that parasites could influence biotransformation, in that this parasite infects liver tissue. Despite anatomical evidence that the livers of infected fish were abnormal with the deposition of much fibrous material, the biotransformation assays indicated that metabolism of the phenol was not impaired.

Perhaps the most important field finding of the present study has been the demonstration that the growth and development of *S. solidus* in sticklebacks are very different at two locations in the River Wandle. If it is hypothesised that some

environmental factor is causal in this context it is clear that, in future, it will be necessary to carry out chronic rather than acute toxicological laboratory studies in an attempt to identify these environmental factors. If it were possible to establish known intensity *S. solidus* infections in sticklebacks in the laboratory, they could then be subjected to a varity of suspected toxicants in a chronic fashion to assess their possible impact on subsequent parasite growth. Unfortunately there is no readily accessable means of generating such infections in the laboratory at present. The general principle of such studies, however, seems a highly useful one and could be attempted on other, more easily culturable helminth parasites.

This multifaceted study is quite unusual in that it has attempted to bring together elements of parasitology and toxicology. In this sense it has been an initial attempt to study the combined effects of two different types of stresses on fish. The study has examined the possible interaction between acute toxicant exposure and parasite infection. It is possible that the combined effects of the two types of stress may only be apparent in fish chronically exposed to toxicants and the differences in susceptibility may only become apparent over the life - cycle of the fish.

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APPENDIX

A. 1: The mean length, prevalence, intensity, mean intensity, mean parasitic index and the mean condition factor of sticklebacks infected with *S. solidus* during the period from December 1992 - till August 1994 at site B.

month	No. of fish	Mean length of fish (cm)	No. of infected fish	% of infected fish (Prevalence)	Mean no.of parasite/infected fish (intensity I)	Mean no. of parasite/fish (including uninfected fish) mean intensity x	Mean parasitic index	Mean condition factor
12.92	27	4.0	27	100.0	4.7	4.7	35.2	1.28
1.93	55	4.1	54	98.2	2.9	2.9	34.4	1.14
2.93	45	4.1	45	100.0	2.8	2.8	44.1	1.12
3.93	15	4.8	15	100.0	2.7	2.7	41.2	1.19
5.93	21	5.0	21	100.0	3.2	3.2	25.6	1.34
6.93	22	2.2	05	22.7	4.0	0.9	25.3	1.43
9.93	54	3.0	31	57.4	1.3	0.7	25.4	1.09
10.93	45	3.6	19	42.2	2.0	0.8	11.6	1.22
11.93	54	3.6	21	38.9	1.3	0.5	30.2	1.19
8.94	59	3.3	23	40.0	1.5	0.6	9.6	1.19

A . 2: The mean length, prevalence, intensity, mean intensity, mean parasitic index and the mean condition factor of sticklebacks infected with *S. solidus* during the period from December 1992 - till August 1994 at site E.

month	No. of fish	Mean length of fish (cm)	No. of infected fish	% of infected fish (Prevalence)	Mean no.of parasite/infected fish (intensity I)	Mean no. of parasite/fish (including uninfected fish) mean intensity x	Mean parasitic index	Mean condition factor
12.92	24	2.8	7	29.2	1.3	0.38	7.1	1.41
1.93	34	3.3	2	5.9	1.0	0.06	11.5	1.11
2.93	26	3.3	2	7.7	1.5	0.12	20.9	1.24
3.93	34	3.2	9	26.5	2.1	0.56	12.5	1.29
5.93	14	3.7	6	42.9	1.5	0.64	9.4	1.34
6.93	10	4.0	2	20.0	1.0	0.20	34.1	1.22
9.93	54	3.2	6	11.1	1.0	0.11	19.2	1.03
10.93	49	2.7	2	4.1	1.0	0.04	12.9	1.07
11.93	46	3.1	3	6.5	1.7	0.11	17.5	1.09
8.94	42	3.0	2	4.8	1.0	0.05	1.0	1.26

A. 3: The total numbers of fish in different size categories at site B.

– : Indicate no fish recovered in these categories.

month	No. of fish	Total no. of fish in different size categories						
		1 - 1.9	2 - 2.9	3 - 3.9	4 - 4.9	5 - 5.9	6 - 6.9	7 - 7.9
12.92	27	-	2	11	13	1	-	-
1.93	55	-	-	27	24	4	-	-
2.93	45	-	-	23	16	5	1	-
3.93	15	-	-	2	9	2	2	-
5.93	21	-	-	2	9	8	2	-
6.93	22	15	3	2	2	-	-	-
9.93	54	4	32	13	2	-	2	1
10.93	45	3	14	15	4	5	4	-
11.93	54	-	19	18	14	1	1	1
8.94	59	3	21	19	8	5	3	-

A. 4: The total numbers of fish in different size categories at site E.

– : Indicate no fish recovered in these categories.

month	No. of fish	Total no. of fish in different size categories						
		1 - 1.9	2 - 2.9	3 - 3.9	4 - 4.9	5 - 5.9	6 - 6.9	7 - 7.9
12.92	24	1	16	4	3	-	-	-
1.93	34	-	11	17	6	-	0	-
2.93	26	-	12	6	8	-	-	-
3.93	34	1	20	6	4	1	2	-
5.93	14	-	3	6	4	1	-	-
6.93	10	1	1	1	6	1	-	-
9.93	54	5	24	13	8	3	1	-
10.93	49	9	23	15	1	1	-	-
11.93	46	1	22	16	3	3	1	-
8.94	42	8	18	5	7	4	-	-

A. 5: The total numbers of *S. solidus* parasites recovered from each fish size categories at site B.

– : indicate no fish recovered in these categories.

month	No. of fish	Total no. of parasite in each host size categories						
		1 - 1.9	2 - 2.9	3 - 3.9	4 - 4.9	5 - 5.9	6 - 6.9	7 - 7.9
12.92	27	-	7	75	43	3	-	-
1.93	55	-	-	73	77	9	-	-
2.93	45	-	-	75	42	9	1	-
3.93	15	-	-	8	20	8	4	-
5.93	21	-	-	16	25	20	7	-
6.93	22	0	1	9	10	-	-	-
9.93	54	2	24	6	3	-	2	2
10.93	45	0	5	10	0	9	13	-
11.93	54	-	6	10	3	3	2	2
8.94	59	0	4	10	13	6	2	-

A. 6: The total numbers of *S. solidus* parasites recovered from each fish size categories at site E.

– : Indicate no fish recovered in these categories.

month	No. of fish	Total no. of parasite in each host size categories						
		1 - 1.9	2 - 2.9	3 - 3.9	4 - 4.9	5 - 5.9	6 - 6.9	7 - 7.9
12.92	24	1	8	0	0	-	-	-
1.93	34	-	1	1	0	-	-	-
2.93	26	-	3	0	0	-	-	-
3.93	34	0	18	0	0	0	1	-
5.93	14	-	1	6	2	0	-	-
6.93	10	0	0	1	1	0	-	-
9.93	54	0	1	3	2	0	0	-
10.93	49	0	2	0	0	0	-	-
11.93	46	0	1	0	0	4	0	-
8.94	42	0	1	1	0	0	-	-

A. 7: Total weight of fish and *S. solidus* (gm), total weight of *S. solidus* alone (gm) and total weight of fish alone (gm) at site B.

month	No. of fish	Total weight of fish + para. (gm)	Total weight of para. alone (gm)	Total weight of fish alone (gm)	Mean para. weight (gm)
12.92	27	33.78	11.94	22.05	0.093
1.93	55	66.12	21.39	44.73	0.135
2.93	45	48.26	9.8	38.46	0.077
3.93	15	31.6	7.89	23.71	0.200
5.93	21	48.93	14.67	34.26	0.216
6.93	22	6.05	1.363	4.687	0.068
9.93	54	29.76	4.901	24.859	0.126
10.93	45	43.82	8.544	35.276	0.231
11.93	54	42.45	6.01	36.44	0.231
8.94	59	42.94	6.44	36.5	0.184

A. 8: Total weight of fish and *S. solidus* (gm), total weight of *S. solidus* alone (gm) and total weight of fish alone (gm) at site E.

month	No. of fish	Total weight of fish + para. (gm)	Total weight of para. alone (gm)	Total weight of fish alone (gm)	Mean para. weight (gm)
12.92	24	8.48	0.117	8.363	0.013
1.93	34	16.05	13	15.92	0.065
2.93	26	13.78	12	13.66	0.040
3.93	34	20.915	375	20.54	0.020
5.93	14	12.03	0.4	11.63	0.044
6.93	10	10.14	0.45	9.69	0.225
9.93	54	24.29	0.79	23.5	0.132
10.93	49	15.07	0.061	15.009	0.031
11.93	46	21.13	0.95	20.18	0.19
8.94	42	22.53	0.13	22.4	0.065

A. 9: The prevalence, intensity and mean intensity of *Gyrodactylus arcuatus* infection in sticklebacks at site B.

month	No. of fish	Total no. of *Gyrodactylus*	No. of infected fish	% of infected fish (Prevalence)	Mean no.of parasite/infected fish (intensity I)	Mean no. of parasite/fish (including uninfected fish) mean intensity x
12.92	27	199	19	70.4	10.5	7.4
1.93	55	81	22	40.0	3.7	1.5
2.93	45	909	44	97.8	20.7	20.2
3.93	15	427	15	100.0	28.5	28.5
5.93	21	539	20	95.2	26.9	25.7
6.93	22	146	17	77.3	8.6	6.6
9.93	54	977	43	79.6	30.5	18.1
10.93	45	393	32	71.1	12.3	8.7
11.93	54	568	32	59.3	17.8	10.5
8.94	59	22	13	22.0	1.7	0.4

A. 10: The prevalence, intensity and mean intensity of *Gyrodactylus arcuatus* infections in sticklebacks at site E.

month	No. of fish	Total no. of *Gyrodactylus*	No. of infected fish	% of infected fish (Prevalence)	Mean no.of parasite/infected fish (intensity I)	Mean no. of parasite/fish (including uninfected fish) mean intensity x
12.92	24	171	19	79.2	9.0	7.1
1.93	34	56	22	64.7	2.6	1.7
2.93	26	544	26	100.0	20.9	20.9
3.93	34	745	31	94.1	24.0	21.9
5.93	14	90	10	71.4	9.0	6.4
6.93	10	254	9	90.0	28.2	25.4
9.93	54	80	28	51.9	2.9	1.5
10.93	49	56	23	46.9	2.5	1.2
11.93	46	120	22	47.8	5.7	2.6
8.94	42	167	16	38.1	10.4	3.9

A. 11: The total numbers of *Gyrodactylus arcuatus* parasites recovered from each fish size categories at site B.

–: Indicate no fish recovered in these categories.

month	No. of fish	Total no. of parasite in each host size categories						
		1 - 1.9	2 - 2.9	3 - 3.9	4 - 4.9	5 - 5.9	6 - 6.9	7 - 7.9
12.92	27	-	8	120	58	13	-	-
1.93	55	-	-	51	30	0	-	-
2.93	45	-	-	607	237	37	28	-
3.93	15	-	-	33	224	23	147	-
5.93	21	-	-	36	189	249	65	-
6.93	22	49	46	41	10	-	-	-
9.93	54	0	534	264	54	-	60	65
10.93	45	9	76	178	42	60	28	-
11.93	54	-	66	343	122	22	11	4
8.94	59	0	1	15	2	2	2	-

A. 12: Shows the total numbers of *Gyrodactylus arcuatus* parasites recovered from each fish size categories at site E.

–: Indicate no fish recovered in these categories.

month	No. of fish	Total no. of parasite in each fish size categories						
		1 - 1.9	2 - 2.9	3 - 3.9	4 - 4.9	5 - 5.9	6 - 6.9	7 - 7.9
12.92	24	0	146	19	6	-	-	-
1.93	34	-	17	30	9	-	-	-
2.93	26	-	191	203	150	-	-	-
3.93	34	0	169	232	279	32	33	-
5.93	14	-	1	60	15	14	-	-
6.93	10	2	1	0	227	24	-	-
9.93	54	2	37	31	8	1	1	-
10.93	49	4	43	11	0	0	-	-
11.93	46	0	19	26	4	71	0	-
8.94	42	1	20	8	135	3	-	-

A. 13: The prevalence, intensity and mean intensity of *Proteocephalus filicollis* infection in sticklebacks at site B.

month	No. of fish	Total no. of *Proteocephalus*	No. of infected fish	% of infected fish (Prevalence)	Mean no.of parasite/infected fish (intensity I)	Mean no. of parasite/fish (including uninfected fish) mean intensity x
12.92	27	32	16	59.3	2.0	1.2
1.93	55	104	32	54.2	3.3	1.8
2.93	45	67	28	62.2	2.4	1.5
3.93	15	86	4	26.7	21.5	5.7
5.93	21	5	3	14.3	1.7	0.2
6.93	22	6	1	4.6	6.0	0.3
9.93	54	12	6	10.2	6.0	0.2
10.93	45	5	4	8.9	1.3	0.1
11.93	54	7	3	5.6	2.3	0.1
8.94	59	15	6	10.2	2.5	0.3

A. 14: The prevalence, intensity and mean intensity of *Proteocephalus filicollis* infection in sticklebacks at site B.

month	No. of fish	Total no. of *Proteocephalus*	No. of infected fish	% of infected fish (Prevalence)	Mean no.of parasite/infected fish (intensity I)	Mean no. of parasite/fish (including uninfected fish) mean intensity x
12.92	24	23	7	29.2	3.3	1.0
1.93	34	1	1	2.9	1.0	0.03
2.93	26	4	2	7.7	2.0	0.1
3.93	34	12	11	32.4	1.1	0.4
5.93	14	0	0	0	0	0
6.93	10	1	1	10	1.0	0.1
9.93	54	0	0	0	0	0
10.93	45	7	4	8.2	1.8	0.1
11.93	46	5	4	10.9	1.3	0.1
8.94	42	2	2	4.8	1.0	0.05

A. 15: The total numbers of *Proteocephalus filicollis* parasites recovered from each fish size categories at site B.

–: Indicate no fish recovered in these categories.

month	No. of fish	Total no. of parasite in each host size categories						
		1 - 1.9	2 - 2.9	3 - 3.9	4 - 4.9	5 - 5.9	6 - 6.9	7 - 7.9
12.92	27	-	3	22	7	0	-	-
1.93	55	-	-	56	39	9	-	-
2.93	45	-	-	49	15	3	0	-
3.93	15	-	-	1	3	0	82	-
5.93	21	-	-	0	3	0	2	-
6.93	22	6	0	0	0	-	-	-
9.93	54	1	10	1	0	-	0	0
10.93	45	0	3	2	0	0	0	-
11.93	54	-	0	7	0	0	0	0
8.94	59	0	0	0	12	2	1	-

A. 16: The total numbers of *Proteocephalus filicollis* parasites recovered from each fish size categories at site E.

–: Indicate no fish recovered in these categories.

month	No. of fish	Total no. of parasite in each host size categories						
		1 - 1.9	2 - 2.9	3 - 3.9	4 - 4.9	5 - 5.9	6 - 6.9	7 - 7.9
12.92	24	0	21	2	0	-	-	-
1.93	34	-	0	0	1	-	-	-
2.93	26	-	3	0	1	-	-	-
3.93	34	0	10	2	0	0	0	-
5.93	14	-	0	0	0	0	-	-
6.93	10	0	0	0	1	0	-	-
9.93	54	0	0	0	0	0	0	-
10.93	49	1	2	4	0	0	-	-
11.93	46	0	3	1	0	1	0	-
8.94	42	0	1	0	0	1	-	-

A. 17: The prevalence, intensity and mean intensity of *Glugea anomala* infection in sticklebacks at site B.

month	No. of fish	Total no. of cyst	No. of infected fish	% of infected fish (Prevalence)	Mean no.of parasite/infected fish (intensity I)	Mean no. of parasite/fish (including uninfected fish) mean intensity x
12.92	27	0	0	0	0	0
1.93	55	0	0	0	0	0
2.93	45	1	1	2.2	1	0.02
3.93	15	0	0	0	0	0
5.93	21	0	0	0	0	0
6.93	22	0	0	0	0	0
9.93	54	0	0	0	0	0
10.93	45	1	1	2.2	1	0.02
11.93	54	4	2	7.4	2	0.07
8.94	59	9	6	15.3	1.5	0.2

A. 18: The prevalence, intensity and mean intensity of *Glugea anomala* infection in sticklebacks at site E.

month	No. of fish	Total no. of cyst	No. of infected fish	% of infected fish (Prevalence)	Mean no.of parasite/infected fish (intensity I)	Mean no. of parasite/fish (including uninfected fish) mean intensity x
12.92	24	17	9	70.8	1.9	0.7
1.93	34	15	10	44.1	1.5	0.4
2.93	26	13	11	50.0	1.2	0.5
3.93	34	13	11	38.2	1.2	0.4
5.93	14	1	1	7.1	1.0	0.07
6.93	10	5	4	50.0	1.3	0.5
9.93	54	9	8	16.7	1.1	0.2
10.93	49	9	6	18.4	1.5	0.2
11.93	46	17	13	36.9	1.2	0.4
8.94	42	14	10	33.3	1.4	0.3

A. 19: Total numbers of *Glugea anomala* parasites recovered from each fish size categories at site B.

–: Indicate no fish recovered in these categories.

month	No. of fish	Total no. of parasite in each host size categories						
		1 - 1.9	2 - 2.9	3 - 3.9	4 - 4.9	5 - 5.9	6 - 6.9	7 - 7.9
12.92	27	-	0	0	1	0	-	-
1.93	55	-	-	0	0	0	-	-
2.93	45	-	-	0	0	0	0	-
3.93	15	-	-	0	0	0	0	-
5.93	21	-	-	0	0	0	0	-
6.93	22	0	0	0	0	-	-	-
9.93	54	0	0	0	0	-	0	0
10.93	45	0	0	0	0	0	1	-
11.93	54	-	0	3	1	0	0	0
8.94	59	0	4	0	1	1	3	-

A. 20: The total numbers of *Glugea anomala* parasites recovered from each fish categories at site E.

–: Indicate no fish recovered in these categories.

month	No. of fish	Total no. of parasite/total fish length (cm)						
		1 - 1.9	2 - 2.9	3 - 3.9	4 - 4.9	5 - 5.9	6 - 6.9	7 - 7.9
12.92	24	1	10	5	1	-	-	-
1.93	34	-	6	6	3	-	-	-
2.93	26	-	5	1	7	-	-	-
3.93	34	1	8	0	1	1	2	-
5.93	14	-	0	0	1	0	-	-
6.93	10	0	0	0	5	0	-	-
9.93	54	0	2	3	3	1	0	-
10.93	49	2	3	3	0	1	-	-
11.93	46	0	2	9	1	4	0	-
8.94	42	0	4	3	4	3	-	-

A 21: Weight of *S. solidus* plerocercoids and the total frequencies at site B and E during the period from December 1992 to August 1994.

Wt. of parasite (mg)	Total frequency E	Total frequency B
1.-99	43	269
100-199	6	207
200-299	6	130
300-399	0	44
400-499	1	20
500-599	0	5
600-699	0	3
700-799	0	1

A 22: Weight of *S. solidus* plerocercoids and the mean frequencies at site B and E during the period from December 1992 to August 1994.

Wt. of parasite (mg)	Mean frequency site E	Mean frequency site B
1.-99	4.3	26.9
100-199	0.6	20.7
200-299	0.6	13
300-399	0	4.4
400-499	0.1	2
500-599	0	0.5
600-699	0	0.3
700-799	0	0.1

A 23: Weight of *S. solidus* plerocercoids and the total numbers of *S. solidus* at site B and E during the period from December 1992 to August 1994.

Wt. of parasite (mg)	Total no. of para. site E	Total no. of para. site B
300-399	0	44
400-499	1	20
500-599	0	5
600-699	0	3
700-799	0	1

A 24: Mean parasite weight at site B and E from December 92 - August 94.

Month	Mean para. wt. site B	Mean para. wt site E
12/92.	0.093	0.013
1/93.	0.135	0.065
2/93.	0.077	0.040
3/93.	0.200	0.020
5/93.	0.216	0.044
6/93.	0.068	0.225
9/93.	0.126	0.132
10/93.	0.231	0.031
11/93.	0.231	0.190
8/94.	0.184	0.065

A 25: Mortalities (%) of unparasitised fish in phenol.

Mortalities of unparasitised fish in phenol			
Conc. mg/l	Exp. no. 1	Exp. no. 2	Exp. no. 3
0	0	0	0
7.5	12.5	12.5	18.8
8	31.3	25	31.3
9	68.8	75	68.8
10	87.5	68.8	81.3
11	93.8	81.3	93.8

A 26: Mortalities (%) of parasitised fish in phenol.

Mortalities of parasitised fish in phenol			
Conc. mg/l	Exp. no. 1	Exp. no. 2	Exp. no. 3
0	0	0	0
7.5	12.5	25	12.5
8	25	37.5	25
9	62.5	62.5	62.5
10	75	75	87.5
11	87.5	87.5	87.5

A 27: Mortalities (%) of unparasitised fish in phenol pre-exposed to salicylamide.

Mortalities of unparasitised fish in phenol pre-exposed to salicylamide		
Conc. mg/l	Exp. no. 1	Exp. no. 2
0	0	0
7.5	25	25
8	37.5	37.5
9	62.5	62.5
10	87.5	75
11	100	87.5

A 28: Mortalities (%) of parasitised fish in phenol pre-exposed to salicylamide.

Mortalities of parasitised fish in phenol pre-exposed to salicylamide		
Conc. mg/l	Exp. no. 1	Exp. no. 2
0	0	0
7.5	25	25
8	37.5	37.5
9	62.5	62.5
10	87.5	87.5
11	100	100

A 29: Mortalities (%) of unparasitised fish in phenol pre-exposed to piperonyl butoxide.

Mortalities of unparasitised fish in phenol pre-exposed to piperonyl butoxide		
Conc. mg/l	Exp. no. 1	Exp. no. 2
0	0	0
7.5	25	25
8	37.5	37.5
9	62.5	62.5
10	87.5	75
11	87.5	87.5

A 30: Mortalities (%) of parasitised fish in phenol pre-exposed to piperonyl butoxide.

Mortalities of parasitised fish in phenol pre-exposed to piperonyl butoxide		
Conc. mg/l	Exp. no. 1	Exp. no. 2
0	0	0
7.5	25	25
8	37.5	37.5
9	62.5	62.5
10	87.5	87.5
11	100	100

A 31: Mortalities (%) of unparasitised fish in pentachlorophenol.

Mortalities of unparasitised fish in pentachlorophenol		
Conc. mg/l	Exp. no. 1	Exp. no. 2
0	0	0
0.1	12.5	12.5
0.2	50	50
0.3	87.5	81.3
0.4	93.8	93.8
0.5	100	100

A 32: Mortalities (%) of parasitised fish in pentachlorophenol.

Mortalities of parasitised fish in pentachlorophenol		
Conc. mg/l	Exp. no. 1	Exp. no. 2
0	0	0
0.1	12.5	25
0.2	50	50
0.3	75	62.5
0.4	87.5	87.5
0.5	100	100